Novel visualization techniques towards identification of atherosclerotic patients at risk

Jager, Nynke

Novel visualization techniques towards identification of atherosclerotic patients at risk

Nynke A. Jager

Studies presented in this thesis were financially supported by the Junior Scientific Masterclass, Groningen University Institute for Drug Exploration (GUIDE) and the Jan Kornelis de Cock Foundation.

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.
Further financial support was generously provided by the Dutch Arthritis Foundation, Biomet, GUIDE, UMCG and the University of Groningen.

Jager, NA

Novel visualization techniques towards identification of atherosclerotic patients at risk

Thesis, University of Groningen, Groningen, the Netherlands

ISBN (printed version): 978-90-367-7398-0
ISBN (electronic version): 978-90-367-7397-3

Cover design by Douwe Oppewal
Type setting and layout by Pieter Jan Karsijns
Printed by NetzoDruk, Groningen

rijksuniversiteit
groningen

Novel visualization techniques towards identification of atherosclerotic patients at risk

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 10 december 2014 om 16.15 uur

door

Nynke Afke Jager

geboren op 30 augustus 1986
te Gaasterlân-Sleat

Promotor
Prof. dr. C.J.A.M. Zeebregts

Copromotores
Dr. M. Bijl
Dr. J. Westra
Dr. H.H. Boersma

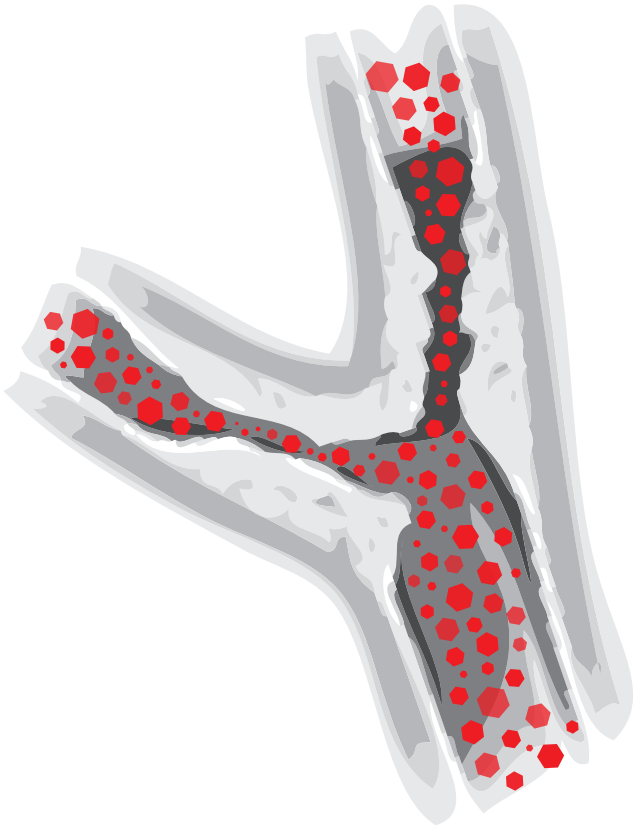
Beoordelingscommissie
Prof. dr. G. Pasterkamp
Prof. dr. T.P.W. Kamphuisen
Prof. dr. R.A.J.O. Dierckx

Paranimfen
R. Hooisma
F. Maas

TABLE OF CONTENTS

CHAPTER 1

Introduction, aim and outline of the thesis



1

Cardiovascular diseases (CVD) such as myocardial infarction or ischemic stroke are inflammatory diseases of the arterial wall characterized by plaque formation. Patients suffering from auto-immune diseases such as rheumatoid arthritis (RA) have an increased risk of developing CVD relative to the general population. RA affects synovial joints and leads to chronic pain, bone erosions and progressive disability. Beyond joint disease, inflammation is the major determinant of accelerated atherosclerosis observed in RA. Mortality risk in RA patients is 1.5 higher than the general population and occurs largely as a result of higher rates of cardiovascular death (1). This might be due to the similarities in the pathogenesis of systemic inflammation and atherosclerosis. In particular macrophage activation and production of pro-inflammatory cytokines are important. This results in endothelial activation and endothelial dysfunction, and eventually leads to development of an atherosclerotic plaque.

Endothelial cell activation is represented by the production of vascular endothelial growth factor (VEGF) and the release of angiopoietin-2 (ANGP-2), which antagonizes binding of angiopoietin-1 (ANGP-1) to the Tie-2 receptor, sensitizing the endothelial cells to up-regulate and release adhesion molecules (2) as soluble vascular cell adhesion molecule-1 (sVCAM-1), thrombomodulin (TM or CD141) and von Willebrand Factor (vWF). Subsequently, endothelial dysfunction will occur which results in increased 'stiffness' of the arterial wall, which can be measured using tonometry of the radial artery by pulse wave analysis (PWA, recalculated to Small Arterial Elasticity (SAE)) (3). Endothelial dysfunction might be regarded as a first sign of endothelial damage and, as such requires recovery. Endothelial progenitor cells (EPCs) appear to be important in endothelial repair (4). EPCs can repair or form new blood vessels through two different mechanisms: by endothelial sprouting from a preexisting capillary network (angiogenesis) or by vasculogenesis, which refers to blood vessel formation from EPCs differentiating *in situ* (5). Therefore, the number and functionality of circulating EPCs may be an important factor contributing to the recovery in the atherogenic process (6). EPCs represent a population of human bone marrow-derived cells expressing CD34, vascular endothelial growth factor receptor-2 (VEGFR-2 or KDR) and CD133, which is a stem cell subset marker (7). Furthermore, advanced glycation end products (AGEs) play a role in the development of atherosclerosis. By interaction with their major receptor (RAGE), AGEs promote inflammatory mechanisms leading to atherosclerotic plaque formation and vulnerability. As such, the above named systemic markers might be useful to measure the cardiovascular risk in patients with auto immune diseases. However, as systemic markers give insight in the cardiovascular status of patients in an early state which can be used in a more preventive matter, risk stratification in later stage is also needed. That is, the presence of the underlying disease itself (subclinical atherosclerosis) is the causal risk factor directly leading to CVD in patients with systemic auto immune diseases.

The chance for an atherosclerotic plaque to cause clinical symptoms depends largely on its probability to become vulnerable and rupture. The term 'vulnerable plaque' was

first coined by Muller *et al.* (8) in 1989, who proposed the concept of non-flow limiting stenosis undergoing acute changes that result in sudden plaque rupture and acute coronary artery thrombosis. However, the debate regarding the substrate responsible for causing symptoms like ischemic stroke or myocardial infarction started much earlier. The thin-cap fibroatheroma (TCFA) is currently considered the prototypic vulnerable plaque phenotype that precedes plaque rupture (9)**.** Nevertheless, plaque vulnerability is not an established medical diagnosis, but an evolving concept to improve risk prediction. Plaque (de)stabilization is a very dynamic process, as it is influenced by many factors such as interaction of inflammatory cells, production of cytokines, and increased degradation of the matrix by matrix metalloproteinases. Nowadays, examination of carotid arteries for intima-media thickness (IMT) is a widely used and non-invasive marker to reveal the severity of atherosclerosis and evaluation of vascular stenosis. However, this conventional diagnostic imaging method is not sufficient for risk stratification. Factors that promote plaque vulnerability (presence of lipid pools and necrotic core with an overlying thin fibrous cap, increased macrophage activity and plaque progression) are observed within plaques that are modest in terms of angiographic stenosis severity within positively remodeled arterial segments (10). So other than anatomical imaging, imaging the cellular and molecular mechanisms of cardiovascular disease has become more important. Next challenge will be to find the optimal imaging modalities and targets. Until now, lipid accumulation, apoptosis and calcification, angiogenesis, thrombosis, proteolysis and inflammation have been targets for innovative imaging techniques (11). By imaging lipid accumulation using technetium labeled LDL (^{99m}Tc-LDL), as well as ^{99m}Tc-oxLDL it was shown that higher uptake of the tracer in carotid plaques was present compared with normal carotid arteries (12,13). Two main contributors to plaque vulnerability are apoptosis and calcification. Apoptosis of macrophages and vascular smooth muscle cells contribute to necrotic core growth, ongoing inflammation and thinning of the fibrous cap. Moreover, apoptotic bodies derived from the macrophages play a role in calcification of the plaque. Targeting annexin A5 has been used to visualize apoptosis. Annexin A5 binds to phosphatidylserine, which is one of the "eat me" signals at the surface of the apoptotic cell (14). Unstable plaques showed higher uptake of annexin A5 while in stable plaques no uptake of annexin A5 was seen after single-photon emission computed tomography (SPECT) imaging (15). Recent studies showed ^{18}F-sodium fluoride targets micro calcification within the necrotic core, which is abundant in a vulnerable plaque. Imaging atherosclerotic plaque using a whole body ^{18}F- sodium fluoride PET/CT scan was described in a recent paper (16). In atherosclerotic plaques, the formation of micro vessels has been studied as a possible contributing factor to plaque destabilization and rupture. Several angiogenic cytokines, such as vascular endothelial growth factor (VEGF) may be potential imaging targets (17). The most promising targets however, are those who target inflammation and/or proteolysis. During the last decade, the glucose analogue [18F]-fluorodeoxyglucose (FDG) in combination with PET imaging has been studied extensively for visualization of

vascular inflammation and identification of vulnerable plaques. [18F]-FDG imaging is based on increased metabolic activity of macrophages, mostly by glucose transporters (GLUT) (18). Proof of principle studies have shown FDG capable of detecting vulnerable plaques in the carotid arteries (19) and evaluate general disease activity in the larger arteries (20). [18F]-FDG PET detects the early stage of foam cell formation and is dependent on inflammation and hypoxia, which are characteristics of plaque vulnerability. Another factor contributing to cap thinning is the involvement of immune cells (especially macrophages), smooth muscle cells (SMCs) and endothelial cells (ECs) by their ability to excrete matrix-degrading proteases such as matrix metalloproteins (MMPs). These enzymes are a result of inflammatory signals in the necrotic core triggering excretion, such as activation by T-lymphocytes trough CD-40L (21), and have the ability to degrade extracellular matrix and collagen in the fibrous cap covering the atherosclerotic lesion, thus making the lesion vulnerable and more prone to rupture. A heterogeneous population of macrophages exists including a classically activated macrophage type (M1) as well as an alternatively activated macrophage population (M2) (22). The M1 macrophage is thought to have pro-inflammatory properties and polarization *in vitro* is driven by interferon gamma and low concentration lipopolysacharride. Macrophages are driven towards the M2 type when the environment includes IL-4 and IL-10. Polarization of macrophages within a plaque is determined by the local micro-environment present in the atherosclerotic lesion (23).

The most promising techniques currently available are radiographic scintigraphic techniques such as PET and SPECT. Combining functional molecular imaging methods with detailed anatomical imaging by HR-MRI or CT seems to be the most useful for diagnostic purposes. Identifying vulnerable plaques before myocardial infarction or stroke occur is therefore the most important goal. The kinetics of inflammatory processes in atherosclerosis and in particular the expression patterns of potential molecular markers within inflamed vessel are still under debate. Targets that may be most promising for clinical imaging applications are monocytes/macrophages or their products (such as MMPs), as hallmarks of inflammations (24). Therefore, in this thesis we tested folate-FITC and ^{99m}Tc-folate as macrophage activity markers, and MMPsense as a marker for MMP activity within an atherosclerotic carotid plaque.

Aim and outline of the thesis

Cardiovascular diseases are related to auto-immune diseases due to similarities in the pathogenesis of systemic inflammation and atherosclerosis. In particular macrophage activation and production of pro-inflammatory cytokines are important. This results in endothelial activation and endothelial dysfunction, and eventually leads to development of an atherosclerotic plaque. So, a systemic marker to measure the cardiovascular risk in patients with auto immune diseases has to be found. Also, the chance for an atherosclerotic plaque to cause clinical symptoms is equal to its probability to destabilize and rupture. Until now, currently established imaging techniques used for making carotid artery disease visible focus on anatomic features of the plaque and give little information on the molecular and cellular processes involved in plaque destabilization. Therefore, they cannot distinguish between stable and unstable plaques prone to rupture. This warrants the need for new imaging modalities that focus on plaque morphology rather than blood vessel narrowing (stenosis) to optimize treatment of atherosclerotic patients. In this thesis, markers for endothelial activation and endothelial dysfunction as a sign for early atherosclerosis correlated to RA-development (part 1), as well as potentially promising markers and tools for imaging atherosclerotic diseases are investigated (part 2).

Part 1 Markers for early atherosclerosis

Systemic inflammation accelerates and causes premature atherosclerotic disease through endothelial activation and dysfunction. Endothelial dysfunction might be regarded as a first sign of endothelial damage and, as such requires recovery. Endothelial progenitor cells (EPCs) appear to be important in endothelial repair. In ***chapter 2*** EPC levels were measured in early RA patients, compared to healthy controls. The aim of this study was to relate EPC numbers to endothelial activation and dysfunction preceding atherosclerosis in early RA patients and to investigate whether a diminished number of EPCs might be associated with increased cardiovascular risk. EPC counts were correlated to disease activity in RA patients (DAS-28), to endothelial activation markers and small arterial elasticity (SAE). Furthermore, endothelial activation markers and SAE were measured at diagnosis and after one year. To evaluate whether reduction of disease activity will influence early markers of cardiovascular disease, these measurements were related to disease activity, independently of traditional risk factors in ***chapter 3.*** Another factor that contributes to the development of atherosclerosis are advanced glycation end products (AGEs). AGEs are formed by non-enzymatic glycation and oxidative reactions. By interaction with their major receptor (RAGE), AGEs have been shown to be related to atherosclerotic plaque formation and vulnerability. AGEs can be measured non-invasively by evaluating skin autofluorescence (skin AF). In ***chapter 4*** skin AF is investigated in relation to plasma levels of MMPs in subjects with a wide range in Framingham risk score profile without overt atherosclerosis, to test whether AGEs and plasma levels of MMPs play a role in the early stages of atherosclerotic plaque development.

Part 2 Imaging of atherosclerosis

Plaque vulnerability and rupture directly leads to CVD. Systemic markers give insight in the cardiovascular status of patients in an early state, but risk stratification in a later stage (imaging subclinical atherosclerosis) is also needed. In ***chapter 5***, the role of macrophage subtypes in systemic inflammation as well as in atherosclerotic disease is depicted. A heterogeneous population of macrophages exists including a classically activated macrophage type (M1) as well as an alternatively activated macrophage population (M2). M2 macrophages might be important as a direct cause of plaque vulnerability by producing tissue degrading MMPs. Folate receptor-β (FR-β; a protein that binds with high affinity to vitamin folic acid) is selectively expressed on activated macrophages, and is thought to be a marker for M2 macrophages. As stated in the above, targets that may be most useful for clinical imaging applications are monocytes/macrophages. Therefore, FR-β is a very promising tool for distinguishing between stable and vulnerable atherosclerotic disease in association with systemic disease. In ***chapter 6*** and ***7*** targeting of FR-β in atherosclerotic carotid plaques is described. Furthermore, macrophage polarization within a plaque is investigated. In ***chapter 6*** we aimed to explore the potential role of fluorescence-labeled folate (folate-FITC) imaging as a macrophage activity marker in the vessel wall. In addition, the distribution of folate–FITC uptake was compared histologically to the presence of activated macrophages and hypoxia (hypoxia-inducible factor–1α (HIF-1α), characteristics of an unstable plaque in the carotid artery. Because of the low penetration depth of folate-FITC, leading to a limited clinical usage, folate imaging using SPECT was investigated in ***chapter 7***. In this study, folate was labeled to technetium (^{99m}Tc). It was evaluated whether this technique can discriminate between a M1-like and M2-like macrophage phenotype within an atherosclerotic carotid plaque. As MMPs are a key factor in plaque vulnerability, their distribution in atherosclerotic plaques is measured by a MMP-sensitive activatable fluorescent probe (MMPSense™ 680) and imaged using a multispectral fluorescence system (IVIS® Spectrum) in ***chapter 8.*** Furthermore, in this chapter the role of smooth muscle cells and macrophage subsets in the production of MMPs is depicted.

Finally, the results of this thesis are summarized and discussed in ***chapter 9***.

1

References

(1) Avina-Zubieta JA, Choi HK, Sadatsafavi M, Etminan M, Esdaile JM, Lacaille D. Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies. Arthritis Rheum 2008 Dec 15;59(12):1690-1697.

(2) Korff T, Ernst E, Nobiling R, Feldner A, Reiss Y, Plate KH, et al. Angiopoietin-1 mediates inhibition of hypertension-induced release of angiopoietin-2 from endothelial cells. Cardiovasc Res 2012 Jun 1;94(3):510-518.

(3) Van Doornum S, McColl G, Jenkins A, Green DJ, Wicks IP. Screening for atherosclerosis in patients with rheumatoid arthritis: comparison of two in vivo tests of vascular function. Arthritis Rheum 2003 Jan;48(1):72-80.

(4) Hristov M, Zernecke A, Liehn EA, Weber C. Regulation of endothelial progenitor cell homing after arterial injury. Thromb Haemost 2007 Aug;98(2):274-277.

(5) Tepper OM, Galiano RD, Kalka C, Gurtner GC. Endothelial progenitor cells: the promise of vascular stem cells for plastic surgery. Plast Reconstr Surg 2003 Feb;111(2):846-854.

(6) Rosenzweig A. Circulating endothelial progenitors--cells as biomarkers. N Engl J Med 2005 Sep 8;353(10):1055-1057.

(7) Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997 Feb 14;275(5302):964-967.

(8) Muller JE, Tofler GH, Stone PH. Circadian variation and triggers of onset of acute cardiovascular disease. Circulation 1989 Apr;79(4):733-743.

(9) Narula J, Nakano M, Virmani R, Kolodgie FD, Petersen R, Newcomb R, et al. Histopathologic characteristics of atherosclerotic coronary disease and implications of the findings for the invasive and noninvasive detection of vulnerable plaques. J Am Coll Cardiol 2013 Mar 12;61(10):1041-1051.

(10) Puri R, Worthley MI, Nicholls SJ. Intravascular imaging of vulnerable coronary plaque: current and future concepts. Nat Rev Cardiol 2011 Mar;8(3):131-139.

(11) Hermus L, van Dam GM, Zeebregts CJ. Advanced carotid plaque imaging. Eur J Vasc Endovasc Surg 2010 Feb;39(2):125-133.

(12) Iuliano L, Signore A, Vallabajosula S, Colavita AR, Camastra C, Ronga G, et al. Preparation and biodistribution of 99m technetium labelled oxidized LDL in man. Atherosclerosis 1996 Sep 27;126(1):131-141.

(13) Lees AM, Lees RS, Schoen FJ, Isaacsohn JL, Fischman AJ, McKusick KA, et al. Imaging human atherosclerosis with 99mTc-labeled low density lipoproteins. Arteriosclerosis 1988 Sep-Oct;8(5):461-470.

(14) Boersma HH, Kietselaer BL, Stolk LM, Bennaghmouch A, Hofstra L, Narula J, et al. Past, present, and future of annexin A5: from protein discovery to clinical applications. J Nucl Med 2005 Dec;46(12):2035-2050.

(15) Kietselaer BL, Reutelingsperger CP, Heidendal GA, Daemen MJ, Mess WH, Hofstra L, et al. Noninvasive detection of plaque instability with use of radiolabeled annexin A5 in patients with carotid-artery atherosclerosis. N Engl J Med 2004 Apr 1;350(14):1472-1473.

(16) Derlin T, Richter U, Bannas P, Begemann P, Buchert R, Mester J, et al. Feasibility of 18F-sodium fluoride PET/CT for imaging of atherosclerotic plaque. J Nucl Med 2010 Jun;51(6):862-865.

(17) Lam MK, Al-Ansari S, van Dam GM, Tio RA, Breek JC, Slart RH, et al. Single-chain VEGF/Cy5.5 targeting vegf receptors to indicate atherosclerotic plaque instability. Mol Imaging Biol 2013 Jun;15(3):250-261.

(18) Dorward DA, Lucas CD, Rossi AG, Haslett C, Dhaliwal K. Imaging inflammation: molecular strategies to visualize key components of the inflammatory cascade, from initiation to resolution. Pharmacol Ther 2012 Aug;135(2):182-199.

(19) Masteling MG, Zeebregts CJ, Tio RA, Breek JC, Tietge UJ, de Boer JF, et al. High-resolution imaging of human atherosclerotic carotid plaques with micro 18F-FDG PET scanning exploring plaque vulnerability. J Nucl Cardiol 2011 Dec;18(6):1066-1075.

(20) Rogers IS, Nasir K, Figueroa AL, Cury RC, Hoffmann U, Vermylen DA, et al. Feasibility of FDG imaging of the coronary arteries: comparison between acute coronary syndrome and stable angina. JACC Cardiovasc Imaging 2010 Apr;3(4):388-397.

(21) Newby AC. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. Physiol Rev 2005 Jan;85(1):1-31.

(22) Medbury HJ, James V, Ngo J, Hitos K, Wang Y, Harris DC, et al. Differing association of macrophage subsets with atherosclerotic plaque stability. Int Angiol 2013 Feb;32(1):74-84.

(23) Wolfs IM, Donners MM, de Winther MP. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost 2011 Nov;106(5):763-771.

(24) Wildgruber M, Swirski FK, Zernecke A. Molecular imaging of inflammation in atherosclerosis. Theranostics 2013 Nov 1;3(11):865-884.

List of abbreviations

[^{18}F]FDG	2-deoxy-2-[^{18}F]fluoro-D-glucose
[^{18}F]FDM	2-deoxy-2-[^{18}F]fluoro-D-mannose
ACEi	antiotensin-converting enzyme inhibitor
ACR	American College of Rheumatology
AF	alkaline phosphatase
AGE	advanced glycation end products
AIIRD	auto-immune inflammatory rheumatic diseases
AMAC-1	alternative macrophage activation-associated CC-chemokine-1
ANGP-1	angiopoietin-1
ANGP-2	angiopoietin-2
Anti-CCP	anti-cyclic citrullinated protein
APC	allophycocyanine
ApoE	apolipoprotein E
ARB	antiotensin receptor blokker
AU	arbitrary units
BMI	body mass index
BSA	bovine serum albumin
CAD	coronary artery disease
CCD	charged coupled device
CCL-1	chemokine (C-C motif) ligand-1
CCR-4	chemokine (C-C motif) receptor-4
CEA	carotid endarterectomy
CFU	colony forming unit
CpG-DNA	C-phosphate-G deoxyribonucleic acid
CRP	C-reactive protein
CS-1	connecting segment-1
Ct	threshold cycle
CT	computed tomography
CTA	computed tomography angiography
CVA	cerebrovascular accident
CVD	cardiovascular disease
CXCL-4,16	chemokine (C-X-C motif) ligand-4,16
CXCR-6	chemokine (C-X-C motif) receptor-6
DAPI	4',6-diamidino-2-phenylindole
DAS-28	disease activity score of 28 joints
DM	diabetes mellitus
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme-linked immunosorbent assay
EPC	endothelial progenitor cell
ECPP	endothelial cells with proliferative potential
FACS	fluorescence activated cell sorting
FcγR	fragment crystallizable gamma receptor
FCM	foam cell macrophages
FCS	fetal calf serum
FDG	fluorodeoxyglucose
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
Flk-1	fetal liver kinase-1
FMT	fluorescence molecular tomography
FOV	field of view
FR-α	folate receptor-alpha
FR-β	folate receptor-beta
FRS	Framingham risk scores
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLUT	glucose transporter proteins
GM-CSF	granulocyte macrophage colony stimulatory factor
GPI	glycosylphosphatidylinositol
CTA	computed tomography angiography
HC	healthy controls
HD	hemodialysis
HDL-cholesterol	high density lipoprotein cholesterol
HIF-1α	hypoxia-inducible factor–1alpha
HLA-DR	human leukocyte antigen-DR
HRP	horseradish peroxidase
ICAM-1	inter-cellular adhesion molecule-1
IFN-γ	interferon-gamma
Il-6, 10, 30, 33	Interleukine-6, 10, 30, 33
IMT	intima media thickness
IRB	institutional review board
iNOS	inducible Nitric Oxide Synthase;
IP-10	interferon-inducible protein 10
KDR	kinase domain region
LDL-cholesterol	low density lipoprotein cholesterol
LPS	lipopolysaccharide
MCP-1	monocyte chemo attractant protein-1
M-CSF	macrophage colony-stimulating factor
MDRD	modification of diet in renal disease

1

MHC	major histocompatibility complex
MI	myocardial infarction
MIP-1	macrophage inflammatory protein-1
MMP	matrix metalloproteinases
MR	mannose receptor
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSOT	multispectral optoacoustic tomography
MTX	methotrexate
NIR	near infrared
NO	nitric oxide
PAD	peripheral artery disease
PASW	predictive analytics software
PBMC	peripheral blood mononuclear cell
PDGF	platelet-derived growth factor
PET	positron emission tomography
PGE2	prostaglandin E2
PWA	pulse wave analysis
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end products
RF	rheumatoid factor
ROI	region of interest
RPMI medium	Roswell Park Memorial Institute medium
RT-PCR	reverse-transcription polymerase chain reaction
SAE	small artery elasticity
SD	standard deviation
SLE	systemic lupus erythematosus
SMC	smooth muscle cell
SPECT	single-photon emission computed tomography
SPSS	statistical package for the social sciences
sVCAM-1	soluble vascular cellular adhesion molecule-1
TBR	target-to-background ratio
TCFA	thin-cap fibroatheroma
TGF-β	transforming growth factor beta
TIA	transient ischaemic attack
TIMP	tissue inhibitors of metalloproteinases
TLR	toll like receptor
TM	thrombomodulin
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor-alpha

TRITC	tetramethyl rhodamine isothiocyanate
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2
VLA-4	very late activation antigen-4
VSMC	vascular smooth muscle cells
v/v	volume fraction
vWF	von Willebrand factor

Part 1

MARKERS FOR EARLY ATHEROSCLEROSIS

CHAPTER 2

Endothelial Progenitor Cells and cardiovascular risk in patients with Early Rheumatoid Arthritis

Nynke A. Jager, Lodewijk de Groot, Steven Wenker, Andries J. Smit, Cees G.M. Kallenberg, Marcel D. Posthumus, Marc Bijl, Johanna Westra

Submitted for publication

Abstract

OBJECTIVES The incidence of cardiovascular disease (CVD) is increased in rheumatoid arthritis (RA) patients. Endothelial progenitor cells (EPCs) are supposed to play a role in repair of endothelial damage. The aim of this study is to relate EPC numbers to endothelial activation and dysfunction preceding atherosclerosis in early RA patients and to investigate whether a diminished number of EPCs might be associated with increased cardiovascular risk.

METHODS EPC numbers were measured from twenty-seven RA-patients, with recent (≤ 1.5 year) onset of disease and 15 healthy controls (HC) by flow cytometry using anti-CD34 and anti-CD133 antibodies. Colony-forming units (CFUs) were counted by culturing mononuclear cells on fibronectin coated plates. Serum endothelial activation markers, small artery elasticity (SAE), disease activity (disease activity score of 28 joints (DAS-28)) and traditional risk factors for atherosclerosis were assessed.

RESULTS Numbers of EPCs, CFU counts and SAE were decreased in RA patients compared to HC ($p=0.02$, $p<0.0001$, and $p=0.02$ respectively), while endothelial activation markers von Willebrand factor (vWF), soluble vascular cell adhesion molecule (sVCAM)-1 and vascular endothelial growth factor (VEGF) were increased ($p=0.0002$, $p=0.05$ and $p=0.04$ respectively). Numbers of EPCs and CFUs were correlated to SAE and inversely correlated to DAS-28 score. EPC numbers were inversely correlated to angiopoietin-2 (ANGP-2) and sVCAM-1 levels.

CONCLUSION CFU counts and EPC numbers are reduced in early RA patients compared to HC, are inversely related to RA disease activity, and correlated to endothelial activation markers and SAE. EPC shortage in peripheral blood might play a role in accelerated development of atherosclerosis in RA patients.

Keywords: Early Rheumatoid Arthritis, Endothelial Progenitor Cells, Colony forming units, Serum endothelial activation markers, Disease Activity Score

Introduction

Rheumatoid arthritis (RA) is a chronic disease characterized by inflammation of joints, but it can also affect a variety of other organ systems (1). RA is associated with an increased mortality. The expected survival of RA patients is likely to decrease by 3-10 years comparing to healthy controls and is related to the severity of the disease and the age of disease onset (2).

The leading cause of death in RA patients is cardiovascular disease (CVD). Cardiovascular mortality is increased in RA patients relative to the general population (1,3). The higher prevalence of atherosclerosis in RA patients is thought to be due to overlapping factors in the pathogenesis of RA and atherosclerosis, such as T-cell activation, macrophage - and mast cell activation, and production of proinflammatory cytokines as TNF-α and IL-6. The systemic inflammatory state results in endothelial activation and endothelial dysfunction (4). However, adequate biomarkers to identify patients at risk for CVD are still needed.

Endothelial cell activation is represented by the production of vascular endothelial growth factor (VEGF) and the release of angiopoietin-2 (ANGP-2), which antagonizes binding of angiopoietin -1 (ANGP-1) to the Tie-2 receptor, sensitizing the endothelial cells to up-regulate and release adhesion molecules (5) as soluble vascular cell adhesion molecule-1 (sVCAM-1), thrombomodulin (TM or CD141) and von Willebrand Factor (vWF). Subsequently, endothelial dysfunction will occur which results in increased 'stiffness' of the arterial wall, measured using tonometry of the radial artery by pulse wave analysis (PWA), which is recalculated to Small Arterial Elasticity (SAE) (6). Endothelial dysfunction might be regarded as a first sign of endothelial damage and, as such requires recovery.

Endothelial progenitor cells (EPCs) appear to be important in endothelial repair (7). EPCs can repair or form new blood vessels through two different mechanisms: by endothelial sprouting from a preexisting capillary network (angiogenesis) or by vasculogenesis, which refers to blood vessel formation from EPCs differentiating *in situ* (8). Therefore, the number and functionality of circulating EPCs may be an important factor contributing to the recovery in the atherogenic process (9). EPCs represent a population of human bone marrow-derived cells expressing CD34, vascular endothelial growth factor receptor-2 (VEGFR-2 or KDR) and CD133, which is a stem cell subset marker (10). There are several techniques to study EPCs. One is detection by cell surface phenotype using fluorescent labeled antibodies and flow cytometry (11). However, this method might fail to discriminate hematopoietic cells from EPCs as some subpopulations of hematopoietic progenitor cells express the same surface markers. For this reason another technique was developed by Hill and co-workers which tests the ability of human peripheral blood mononuclear cells (PBMCs) to develop into colony-forming units (CFUs). PBMCs were plated on fibronectin-coated dishes; next nonadherent cells were replated to quantify the emergence of CFU (called CFU-Hill) seven days later (12). This *in vitro* adhesion and growth method exhibit more endothelial characteristics (expression of CD31, Tie-2) and is therefore more specific,

but also more complex and time-consuming than flow cytometry. In the present study both methods were used.

The aim of this study is to evaluate whether the increased endothelial activation and dysfunction of RA patients is related to decrease in EPC numbers. We hypothesize that in RA patients, in comparison to healthy controls, EPC numbers in the peripheral blood are lower due to disease activity. Subsequently, endothelial repair mechanisms are less active and lead to endothelial dysfunction, reflected by a decrease in SAE and an increase in traditional risk factors for atherosclerosis.

Materials and Methods

Study design

RA patients have a higher risk to develop cardiovascular disease over time (13). To prevent confounding effects of longstanding disease patients with recent onset RA (disease duration ≤ 1.5 year) as well as healthy controls (HC) were included between December 2010 and October 2011. Patients with diagnosis based on the American College of Rheumatology criteria for RA (14) were asked to participate. Exclusion criteria were diabetes mellitus (fasting blood glucose level >7 mmol/L, or use of antidiabetic drugs), pregnancy, renal impairment (serum creatinin >140 µmol/L), surgery in the past three months or a history of CVD. Healthy controls were recruited from hospital personnel and relatives from the researchers. Traditional cardiovascular risk factors such as smoking status, obesity (body mass index, BMI), hyperlipidemia and hypertension were recorded. Hypertension was defined as systolic arterial pressure above 140 mmHg and/or diastolic arterial pressure above 90 mmHg, or use of antihypertensive drugs prescribed with the aim to reduce blood pressure (15). Hyperlipidemia was diagnosed if plasma total cholesterol exceeded 6.21 mmol/l, plasma LDL cholesterol exceeded 3.36 mmol/l, plasma triglycerides exceeded 2.26 mmol/l, or current use of lipid-lowering drugs, similar to Brevetti *et al.* (15). Also the IMT (intima media thickness) of the carotid artery was measured by duplex ultrasonography at three locations, namely one centimeter proximal of the carotid bifurcation, on the bulbus carotis and one centimeter distal of the bulbus in the internal carotid artery, as a measure for degree of stenosis (16). The DREAM protocol was used for treatment of RA patients (17). In RA patients, disease activity was assessed using the Disease Activity Score for 28 joints (DAS-28 score) (18). The study was approved by the local medical ethics committee of the University Medical Center of Groningen and informed written consent was obtained from all participants.

Endothelial progenitor cells measured by Flow Cytometry analysis

Two hundred microliters of lithium-heparin blood was incubated with anti-CD34-PE (clone 581, IQproducts, Groningen, The Netherlands) and anti-CD133-APC (clone AC133, Miltenyi Biotec, Auburn, USA) or appropriate isotype controls. To lyse erythrocytes and fix remaining

cells, samples were incubated with FACS lysing solution (BD Bioscience) using a dilution of 1:10. PBS with 1% BSA was used to wash the cells. Flow cytometry measurements were performed on a BD Bioscience FACS Calibur flow cytometer, and analyzed using Winlist 6.0**.**

Endothelial progenitor cell quantification by CFU-Hill assay

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Axis Shield PoC As, Oslo, Norway). Subsequently, cells were washed with RPMI Medium 1640 (Lonza, Walkersville, MD, USA) + Gentamycin and suspended in culture medium consisting of RPMI supplemented with 20% v/v fetal calf serum (both from BioWhittaker, Verviers, Belgium), 2 mM L-glutamine (GIBCO Products, Invitrogen, Breda, The Netherlands), 5 U/ml heparin (LEO Pharma, Ballerup, Denmark), 1% v/v PenStrep (Sigma, Zwijndrecht, The Netherlands), 50 µg/ml bovine brain extract (FBS, GIBCO/Invitrogen, CA). The cells were plated on fibronectin-coated plates at 5 million cells per well and incubated at 37 °C and 5% CO_2. After 48 hours, non-adherent cells were harvested and diluted to a concentration of 1 million cells per well, and replated to a fibronectin-coated 24-well plate and cultured for 7 days. Medium was changed at day 3 and 6. CFUs were visually counted in quadruplo (4 wells) using an inversion microscope by two independent observers (NaJ, JW). The average of this count was used for statistical analysis.

Quantification of endothelial activation markers by ELISA

Serum levels of sVCAM-1, VEGF, ANGP-2 and TM were measured by ELISA according to the manufacturer's instructions (R&D Systems, Abingdon, UK). For determination of vWF we used an in house ELISA as previously described (19).

Small Arterial Elasticity measurement

In all patients and HC SAE was determined non-invasively using pulse-wave analysis measurement (CR-2000, Hypertension Diagnostics, Eagan, MN, USA), which is described previously (20).

Intima Media Thickness

The degree of stenosis of the carotid artery was measured at three locations, namely one centimeter proximal of the carotid bifurcation, on the bulbus carotis and one centimeter distal of the bulbus in the internal carotid artery by duplex ultrasonography using an Acuson 128XP ultrasound system with 7 MHz linear array transducers (Acuson Corp., Silicon Valley, California, USA) (21). A B-mode image was obtained after which a probe was positioned perpendicular to the far wall, showing an intima-media complex over approximately one centimeter. Mean IMT (the mean of the segment studied) and the maximum IMT (the highest IMT value found among the segment studied) were determined. As endpoint we used the mean of the mean (mean IMT) of the far wall IMT of the six imaged carotid

segments.

Statistical analysis

Values are presented as mean ± standard deviation or median (range) when appropriate, unless stated otherwise. The sample size of 29 patients was based on an earlier study, in which the sample size was calculated on the assumption that RA patients who achieved remission would normalize in SAE from 4.5 to 7.7 ml/mmHg100, based on a SD of 3.7 (6). For correlations, Pearson's and Spearman's correlation coefficients were used when appropriate. Non-paired continuous variables with a non-parametric distribution were analyzed using the Mann-Whitney U-test. The chi-square test was used for categorical variables and for small expected frequencies the Fischer's exact test was used. To control for influence of differences in cardiovascular risk factors between patients and HC, multivariate linear regression analysis for correlations with EPC and CFU levels was performed by using forward inclusion of values of baseline characteristics with P values ≤0.10 in univariate analysis. The same was done to investigate the biomarker with the strongest association with EPC and CFU counts. A two-sided P value ≤ 0.05 was considered statistically significant. Statistical tests were done with the Statistical Package for the Social Sciences (SPSS statistics version 20.0, SPSS inc®, Chicago IL, USA).

Results

Patient characteristics

A total of 27 early RA patients, mean age 58 ± 9 years, and 15 HC, mean age 48 ± 13 years, were included. Of 29 included early RA patients, two patients were excluded; one due to a history of coronary artery disease (CAD) and one due to a myocardial infarction (MI). Traditional cardiovascular risk factors such as BMI, hyperlipidemia, hypertension and CRP of participants are shown in Table 1.

Table 1. Baseline characteristics

	Patients (n=27)	**HC (n=15)**	**P value**
Men, n (%)	6 (22)	6 (40.0)	NS
Age, years	58 ± 9	48 ± 13	0.01
BMI, kg/m^2	26.7 (19-37)	24 (20-33)	NS
Hypertension, n (%)	16 (59)	2 (13.3)	0.002
Systolic blood pressure, mmHg	140.3 ± 24	126.9 ± 13	0.03
Diastolic blood pressure, mmHg	80.2 ± 11	70.5 ± 7	0.02
Dyslipidemia, n (%)	7 (25.9)	3 (21.4)	NS
LDL	3.6 ± 1	3.6 ± 0.7	NS
HDL	1.6 ± 0.4	1.7 ± 0.4	NS
Triglycerides mmol/L	1.1 ± 0.4	1.1 ± 0.5	NS
HbA1c (%)	5.7 (4.7-6.6)	5.8 (4.6-6)	NS
CRP, mg/L	5.0 (0.1-65)	1.1 (0.2-5.1)	0.03
DAS-28	2.4 (1.7-6.2)		
Disease duration (months)	12 (11-16)		
RF, iU/mL	87 (8-1180)		
Positive (> 25 iU/mL), n (%)	17 (62.9)		
Anti-CCP, U/mL	63 (1-340)		
Positive (> 10 U/mL), n (%)	22 (81.5)		
No immunosuppressives, n (%)	4 (13.8)		
Methotrexate, n (%)	18 (66.6)		
Median (range), in users (mg/day)	15 (7.5–25)		
Other immunosuppressive drugs, n (%)	6 (22.2)*		

* Three patients used Adalimumab, one patient was on Rituximab and two patients used prednisone. Hypertension was defined as: systolic blood pressure > 140mmHg, diastolic blood pressure > 90mmHg or use of antihypertensive drugs. Dyslipidemia was defined as: total cholesterol > 6.2 mmol/l, LDL > 3.2 mmol/l, triglycerides > 2.26 mmol/l or use of lipid lowering drugs. Unless otherwise indicated, data are expressed as mean ± standard deviaton or median (range) when appropriate; percentages between brackets.

Medication use, presence of rheumatoid factor (RF), anti-CCP and DAS-28 score of RA patients are given as well. In the RA patients group, five patients were smokers and seven patients reported a history of smoking. In the HC group none of the participants reported smoking. Five RA patients used statins. In HC no history of cardiovascular disease was reported and no statins were used. The mean age of the RA patients was 10 years higher compared to the HC group. However, in univariate analysis age was not related to EPC counts (p=0.628). Age was not significantly related to CFU in univariate analysis (p=0.090), in contrast to smoking (p<0.001) and CRP (p=0.023). In multivariate analysis including age, smoking and CRP, only smoking was independently associated to CFU counts (p=0.002). No relation could be found between dosage of MTX, used in the majority of patients, and EPC numbers and CFU counts. The same yields for usage of other immunosuppressives and EPC numbers and CFU counts.

Endothelial progenitor cells

EPC numbers, determined by FACS analysis ($CD34^+/CD133^+$ cells), in RA patients were significantly lower compared to those in HC (481.4 ± 292.7 vs 715.4 ± 312.3, P=0.02, Figure 1A). The same applied for CFU counts (3.4 ± 2.3 vs 7.7 ± 1.9, P<0.0001, Figure 1B), suggesting impaired endothelial progenitor cell numbers in RA patients compared to HC. No correlation between EPCs and CFUs was found in RA (r=0.38, p=0.07) nor in HC (r=0.38, p=0.20, Figure 1C).

Figure 1.

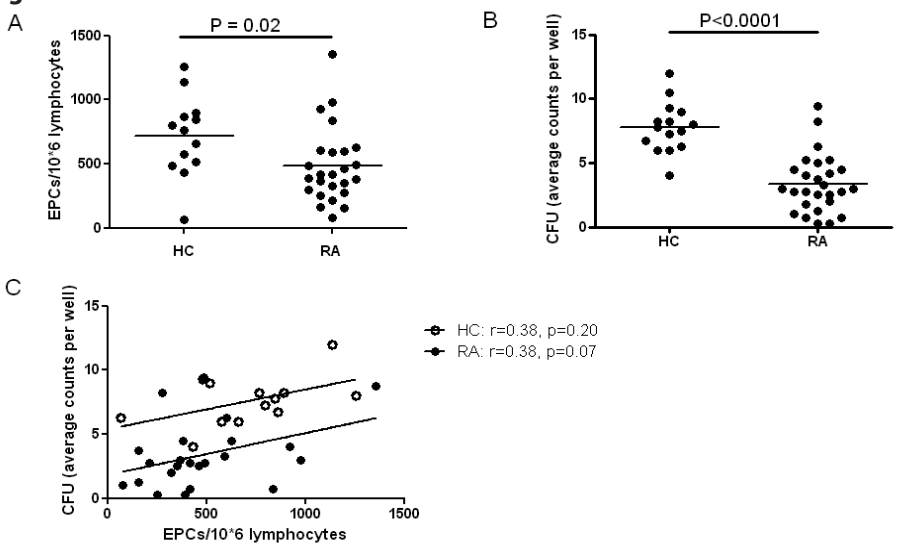


Figure 1. EPC numbers in RA patients and healthy controls. (A) $CD34^+/CD133^+$ cells of RA patients were significantly lower compared to EPC numbers of HC (p=0.02). (B) CFU counts were significantly lower in RA patients compared to healthy controls. (C) Correlation between CFU counts and EPC numbers.

Strikingly, a significant negative correlation was seen between RA disease activity DAS-28 and both EPC and CFU numbers (r=-0.49, p=0.03, Figure 2A and r=-0.58, p=0.008, Figure 2B respectively).

Figure 2.

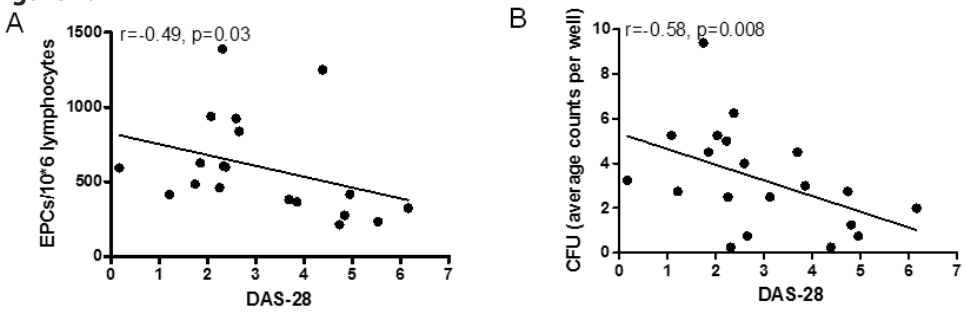


Figure 2. EPC numbers compared to disease activity DAS-28 score in RA patients and endothelial activation markers in healthy controls and RA patients. (A) EPC numbers were negatively correlated to DAS-28 (r=-0.49, p=0.03) (B) CFU counts were negatively correlated to DAS-28 (r=-0.58, p=0.008)

Endothelial activation markers

Measurement of sVCAM-1, VEGF, ANGP-2, TM and vWf levels showed that vWF, sVCAM-1 and VEGF were significantly increased in RA patients compared to HC (192.2 ± 75.8 ng/ml vs 100.2 ± 50.1 ng/ml, P=0.0002, Figure 3A; 387.6 ± 191.9 ng/ml vs 293.6 ± 49.8 ng/ml, P=0.05, Figure 3B; and 149.9 ± 84.6 pg/ml vs 93.8 ± 87.6 pg/ml, P=0.04, Figure 3C respectively).

Figure 3.

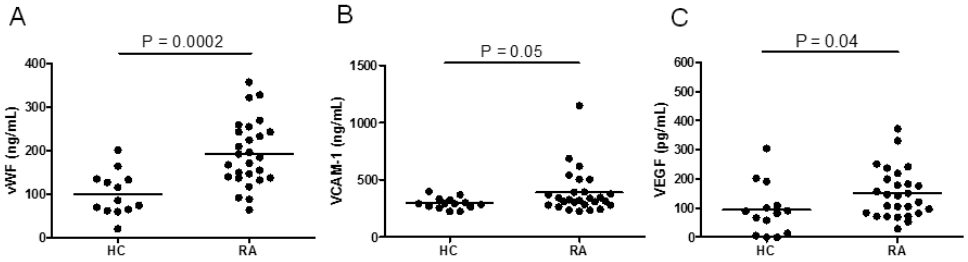


Figure 3. (A) vWF is increased in RA patients compared to HC (p=0.0002) (B) sVCAM-1 is increased in RA patients compared to HC (p=0.05) (C) VEGF is increased in RA patients compared to HC (p=0.04).

ANGP-2 and TM levels were increased in RA patients compared to HC as well, but did not reach significance (902.9 ± 718.3 pg/ml vs 759.5 ± 497.5 pg/ml and 3.9 ± 1.3 ng/ml vs 3.7 ± 0.7 ng/ml, respectively). Interestingly, significant negative correlations were found in RA patients, but not in HC, between sVCAM-1 levels and EPC numbers (r=-0.58, p=0.004) and ANGP-2 levels and EPC numbers (r=-0.60, p= 0.01). In univariate analysis, sVCAM, ANGP-2 and SAE were related to EPC measurement, and vWF and SAE were correlated to CFU count

(Table 2). Multivariate analysis showed that only sVCAM was independently associated with EPC (B: -1,456 (95% CI: -2635- -0.277), p=0.017), while only SAE was independently associated with CFU counts (B: 5.193 (95% CI: 0.960 – 9.425), p=0.018).

Table 2. Association between EPC and CFU measurement, serum markers and SAE

	Univariate analysis	
	B (95% CI)	**P-value**
A. *relation with EPC*		
sVCAM-1, ng/ml	-1.561 (-2.840 -0.282)	0.018*
VEGF,	-1.186 (-3.154 0.782)	0.229
ANGP-2, pg/ml	-0.269 (-0.484 -0.054)	0.016*
TM, ng/ml	42.468 (-145.322 230.257)	0.648
vWf, ng/ml	-0.754 (-2.514 1.006)	0.389
SAE, ml/mmHg	532.809 (16.737 1048.881)	0.043*
B. *relation with CFU*		
sVCAM-1, ng/ml	-0.004 (-0.010 0.002)	0.151
VEGF,	-0.004 (-0.014 0.007)	0.518
ANGP-2, pg/ml	-0.001 (-0.002 0.001)	0.462
TM, ng/ml	0.522 (-0.316 1.361)	0.215
vWf, ng/ml	-0.014 (-0.025 -0.002)	0.019*
SAE, ml/mmHg	4.682 (0.580 8.783)	0.026*

Multivariate regression analysis was performed by using markers with P values ≤0.10* in univariate analysis, to elucidate the biomarker with the strongest association with EPC and CFU counts. SAE was log transformed.

Small artery elasticity

SAE was decreased in RA patients compared to HC, as shown in Figure 4A (P=0.02), indicating increased stiffness of arteries in RA patients. Strikingly, SAE was significantly correlated to EPC numbers (Figure 4B, r=0.40, p=0.02) and to CFU counts (r=0.35, p=0.04). However, when RA patients and HC were evaluated separately, no significant correlation remained (EPC numbers, Figure 4C).

Figure 4.

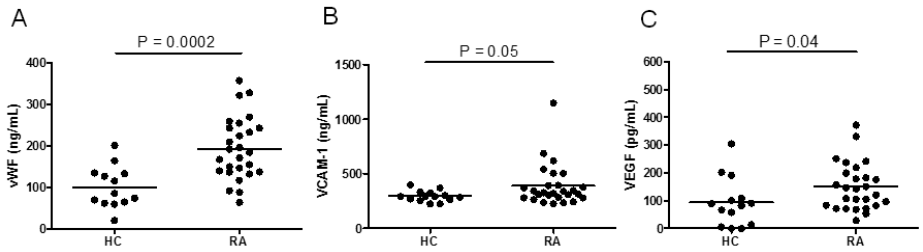


Figure 4. Vascular measurements in RA patients and healthy controls. (A) Small Arterial Elasticity was significantly reduced in RA patients (4.0 ± 1.5 ml/mmHg) compared to healthy controls (6.9 ± 3.8 ml/mmHg). (B) SAE was positively correlated to EPC counts (r=0.40, p=0.02). (C) No significant correlation remained when RA patients and HC were evaluated separately.

Intima Media Thickness

IMT measurements showed no significant difference in the degree of stenosis of healthy controls and RA patients (p=0.19, 0.74 ± 0.2 vs 0.98 ± 0.5 mm respectively). No correlation was found between IMT and neither EPC nor CFU measurement.

Discussion

The current study shows that circulating EPC and CFU counts, as well as SAE, are reduced in early RA patients compared to healthy controls, while endothelial activation markers vWF, sVCAM-1 and VEGF were increased. Furthermore, EPC and CFU counts were correlated to SAE and inversely related to endothelial activation markers, such as VCAM-1, ANGP-2, and to disease activity. These data suggest that decreased numbers of EPCs in peripheral blood might play a role in accelerated development of atherosclerosis in RA patients. The results remained significant after correcting for the differences in baseline characteristics between both groups in prevalence of pre-existing cardiovascular risk factors and age (Table 1).

Controversy still exists about the exact definition, identity and phenotype of EPCs. Many different types of blood cells and endothelial cells are being included in the term EPC. Although cells co-expressing $CD34^+$, VEGFR2 (KDR) and $CD133^+$ are commonly considered to be EPCs (22), additional markers such as CD45, flk-1, Tie-2 and CD146 are considered markers of EPCs as well. To test the population of $KDR^+/CD34^+/CD133^+$ cells, a minimum

amount of events is needed to identify an adequate number of this scarce population (<0.01% of PBMCs without enrichment) using flow cytometry. Two hundred microliters of peripheral blood was not enough to detect these triple marked cells. As adding KDR to the analysis of cells increases specificity of analysis, this can be seen as a limitation of our study (22). To improve the detection sensitivity, we identified EPCs as $CD34^+/CD133^+$ (23,24).

Some experts consider broadening the definition of an EPC to: "hematopoietic lineage cells that display proangiogenic properties" (25). Given the controversy mentioned we deliberately chose to include the spindle like cells that grow in a CFU-Hill to determine EPCs as well. One reason for the lack of correlation between the two methods could be the possibility that the different methods sample EPCs at different stages of differentiation (26). Furthermore, new insights show CFU-Hill reflect a variety of blood cells including monocytes, lymphocytes and hematopoietic progenitor cells skewed towards the myeloid lineage, and therefore express different cell types as EPCs measured by flowcytometry. The fact that CFU-Hill fails to exclude the presence of hematopoietic cells in the colonies and do not directly demonstrate vessel forming ability is a disadvantage of this method (25).

The observation that disease activity in RA patients, measured by DAS-28, shows a correlation with EPC and CFU counts has been demonstrated before (27). However, in the latter study RA patients with mean disease duration of 10 years were included, while we included early RA patients (with a disease duration ≤ 1.5 year). By doing so, long term influences of medication were reduced to a minimum. To our knowledge, our study is the first to show that EPC and CFU counts are associated with endothelial activation, namely, ANGP-2 and sVCAM-1 levels were inversely correlated to EPC numbers. In a retrospective study we previously demonstrated increased levels of VEGF and ANGP-2 in a large group of recent-onset RA patients (28). These markers were shown to be highly correlated to inflammation and disease activity, whereas RA patients who developed cardiovascular disease had significantly higher ANGP-2 levels than those who did not.

As mentioned, negative correlations were found in RA patients between sVCAM-1 levels and EPC numbers. Also, sVCAM-1 levels were higher in RA patients compared to HC, and independently associated with EPC numbers. sVCAM-1 induces firm adhesion of inflammatory cells at the vascular surface and contributes to endothelial dysfunction, reflected by SAE, which is a major factor in cardiovascular disease. As SAE was independently associated with CFU counts, this indicates that sVCAM-1 might be a good marker for predicting cardiovascular risk in chronic inflammatory diseases such as rheumatoid arthritis (29).

Previous research has shown that RA patients with active disease suffer earlier from cardiovascular disease (30) and that EPC levels are decreased in RA patients (27), as well as in systemic lupus erythematosus (SLE) patients (31,32), which is in concordance with our findings. A possible explanation for the lower EPC numbers in inflammatory diseases could be that these cells migrate to other tissues, for instance to the inflamed synovial membrane. This hypothesis is supported by the work of Rüger *et al*. who found an enrichment of EPCs

in the inflamed joint (33). In a collagen-induced arthritis model Silverman *et al.* showed selective recruitment of EPCs to inflamed joint tissue, demonstrating a role for the VCAM-1/ VLA-4 (very late activation antigen) system in directing EPCs to synovial fibroblasts (34). In a recent paper Isozaki *et al* showed that CXCL16 and its receptor CXCR6 are a central ligand receptor pair that induce EPC recruitment and blood vessel formation in the RA joint (35).

An alternative explanation could be that EPC numbers are decreased due to continuous endothelial damage, as occurs in patients with muscular dystrophy. In this disease, the satellite cells, a type of progenitor cells in skeletal muscle, get exhausted. Furthermore, cells that do remain in these patients show evidence of accelerated ageing (36). Decrease in EPCs might also be due to use of immunosuppressive drugs. However, no relation could be found between dosage of MTX, used in the majority of patients, and EPC numbers. The observation of rising EPCs numbers when disease activity decreases, strongly argues against suppressive effects of anti-rheumatic drugs. Nevertheless, further research to examine the exact role of anti-rheumatic drugs on EPC numbers is necessary.

In contrast to our findings, De Villeroché *et al.* indicated that EPC levels are increased in RA patients due to disease activity. They suggested that this is due to different factors, including inflammation, vascular injury, and potentially the immune response with bone marrow changes preceding inflammation in the synovium (37). However, the mean disease duration in this study was sixteen years, a different subpopulation of EPCs was used and patients with conventional cardiovascular risk factors were excluded, so these data cannot be compared to the present study. The same applies to the study of Rodríguez-Carrio *et al.* who found increased EPCs in SLE patients, but used another subpopulation of EPCs (mature EPCs ($CD34^+VEGFR2^+$) and ECs ($VEGFR2^+$)) (38).

It is of interest to investigate whether the decrease in EPC numbers is typical for inflammatory diseases with cardiovascular involvement, or whether it also occurs in other diseases. The group of Kim *et al.* investigated the number of circulating EPCs in gastric and breast cancer patients. Although the plasma levels of VEGF (mobilization of EPCs from the bone marrow) were elevated, there was no increase in EPC number (39). However, in a study with type two diabetes patients, a generalized decrease in progenitor cell populations was observed. This reduction was, consistent with our study, negatively associated with disease severity (40) and suggests EPCs are lowered in various diseases with cardiovascular involvement. Patients with diabetes were excluded to participate in the study, to prevent a bias in EPC measurement as a result of the disease. Previous work showed that a reduction in EPC number could be a consequence of ageing (41). In our study, the mean age in the patient group was 10 years higher compared to the HC group. No significant association with age could be found. However, the number of study participants is limited for subgroup analyses, which is a major limitation to this study. As EPCs are important in endothelial repair and are related to endothelial activation markers and disease activity, but not to established atherosclerosis (measured by IMT), their reduced numbers in RA patients are in

agreement with increased cardiovascular mortality in these patients. Furthermore, these data suggest that EPC count might serve as a very early biomarker in the atherosclerotic process in subjects with rheumatoid arthritis.

Conclusions

CFU counts and EPC numbers are reduced in early RA patients, are inversely related to RA disease activity, and correlate to endothelial activation markers and endothelial dysfunction. Measurement of endothelial progenitor cells by flowcytometry or CFU culture might be of value in cardiovascular risk assessment in RA patients (25) as EPC counts may represent a balance between the magnitude of injury and the capacity for endothelial cell repair (12). Still, it is not clear whether reduced EPC numbers directly contribute to the pathophysiology of atherosclerosis, and whether the decreased numbers found in early RA patients is a consequence of influx in synovial tissue in RA or results from medication use. To answer these questions further studies, including assessment of functionality of EPCs in RA patients and their link with clinical events, are needed.

References

(1) Minaur NJ, Jacoby RK, Cosh JA, Taylor G, Rasker JJ. Outcome after 40 years with rheumatoid arthritis: a prospective study of function, disease activity, and mortality. J Rheumatol Suppl 2004 Mar;69:3-8.
(2) Alamanos Y, Drosos AA. Epidemiology of adult rheumatoid arthritis. Autoimmun Rev 2005 Mar;4(3):130-136.
(3) Watson DJ, Rhodes T, Guess HA. All-cause mortality and vascular events among patients with rheumatoid arthritis, osteoarthritis, or no arthritis in the UK General Practice Research Database. J Rheumatol 2003 Jun;30(6):1196-1202.
(4) Libby P. Role of inflammation in atherosclerosis associated with rheumatoid arthritis. Am J Med 2008 Oct;121(10 Suppl 1):S21-31.
(5) Korff T, Ernst E, Nobiling R, Feldner A, Reiss Y, Plate KH, et al. Angiopoietin-1 mediates inhibition of hypertension-induced release of angiopoietin-2 from endothelial cells. Cardiovasc Res 2012 Jun 1;94(3):510-518.
(6) Van Doornum S, McColl G, Jenkins A, Green DJ, Wicks IP. Screening for atherosclerosis in patients with rheumatoid arthritis: comparison of two in vivo tests of vascular function. Arthritis Rheum 2003 Jan;48(1):72-80.
(7) Hristov M, Zernecke A, Liehn EA, Weber C. Regulation of endothelial progenitor cell homing after arterial injury. Thromb Haemost 2007 Aug;98(2):274-277.
(8) Tepper OM, Galiano RD, Kalka C, Gurtner GC. Endothelial progenitor cells: the promise of vascular stem cells for plastic surgery. Plast Reconstr Surg 2003 Feb;111(2):846-854.
(9) Rosenzweig A. Circulating endothelial progenitors--cells as biomarkers. N Engl J Med 2005 Sep 8;353(10):1055-1057.
(10) Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997 Feb 14;275(5302):964-967.
(11) Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. Arterioscler Thromb Vasc Biol 2008 Sep;28(9):1584-1595.
(12) Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003 Feb 13;348(7):593-600.
(13) Avina-Zubieta JA, Choi HK, Sadatsafavi M, Etminan M, Esdaile JM, Lacaille D. Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies. Arthritis Rheum 2008 Dec 15;59(12):1690-1697.
(14) Kay J, Upchurch KS. ACR/EULAR 2010 rheumatoid arthritis classification criteria. Rheumatology (Oxford) 2012 Dec;51 Suppl 6:vi5-9.
(15) Brevetti G, Silvestro A, Schiano V, Chiariello M. Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index. Circulation 2003 Oct 28;108(17):2093-2098.
(16) Nienhuis HL, de Leeuw K, Bijzet J, van Doormaal JJ, van Roon AM, Smit AJ, et al. Small artery elasticity is decreased in patients with systemic lupus erythematosus without increased intima media thickness. Arthritis Res Ther 2010;12(5):R181.
(17) Vermeer M, Kuper HH, Hoekstra M, Haagsma CJ, Posthumus MD, Brus HL, et al. Implementation of a treat-to-target strategy in very early rheumatoid arthritis: results of the Dutch Rheumatoid Arthritis Monitoring remission induction cohort study. Arthritis Rheum 2011 Oct;63(10):2865-2872.
(18) Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995 Jan;38(1):44-48.
(19) de Leeuw K, Sanders JS, Stegeman C, Smit A, Kallenberg CG, Bijl M. Accelerated atherosclerosis in patients with Wegener's granulomatosis. Ann Rheum Dis 2005 May;64(5):753-759.

(20) de Groot L, Hinkema H, Westra J, Smit AJ, Kallenberg CG, Bijl M, et al. Advanced glycation endproducts are increased in rheumatoid arthritis patients with controlled disease. Arthritis Res Ther 2011 Dec 14;13(6):R205.
(21) Nienhuis HL, de Leeuw K, Bijzet J, van Doormaal JJ, van Roon AM, Smit AJ, et al. Small artery elasticity is decreased in patients with systemic lupus erythematosus without increased intima media thickness. Arthritis Res Ther 2010;12(5):R181.
(22) Distler JH, Allanore Y, Avouac J, Giacomelli R, Guiducci S, Moritz F, et al. EULAR Scleroderma Trials and Research group statement and recommendations on endothelial precursor cells. Ann Rheum Dis 2009 Feb;68(2):163-168.
(23) Allanore Y, Batteux F, Avouac J, Assous N, Weill B, Kahan A. Levels of circulating endothelial progenitor cells in systemic sclerosis. Clin Exp Rheumatol 2007 Jan-Feb;25(1):60-66.
(24) Rafii S. Circulating endothelial precursors: mystery, reality, and promise. J Clin Invest 2000 Jan;105(1):17-19.
(25) Richardson MR, Yoder MC. Endothelial progenitor cells: quo vadis? J Mol Cell Cardiol 2011 Feb;50(2):266-272.
(26) George J, Shmilovich H, Deutsch V, Miller H, Keren G, Roth A. Comparative analysis of methods for assessment of circulating endothelial progenitor cells. Tissue Eng 2006 Feb;12(2):331-335.
(27) Grisar J, Aletaha D, Steiner CW, Kapral T, Steiner S, Seidinger D, et al. Depletion of endothelial progenitor cells in the peripheral blood of patients with rheumatoid arthritis. Circulation 2005 Jan 18;111(2):204-211.
(28) Westra J, de Groot L, Plaxton SL, Brouwer E, Posthumus MD, Kallenberg CG, et al. Angiopoietin-2 is highly correlated with inflammation and disease activity in recent-onset rheumatoid arthritis and could be predictive for cardiovascular disease. Rheumatology (Oxford) 2011 Apr;50(4):665-673.
(29) Constans J, Conri C. Circulating markers of endothelial function in cardiovascular disease. Clin Chim Acta 2006 Jun;368(1-2):33-47.
(30) Fischer LM, Schlienger RG, Matter C, Jick H, Meier CR. Effect of rheumatoid arthritis or systemic lupus erythematosus on the risk of first-time acute myocardial infarction. Am J Cardiol 2004 Jan 15;93(2):198-200.
(31) Castejon R, Jimenez-Ortiz C, Valero-Gonzalez S, Rosado S, Mellor S, Yebra-Bango M. Decreased circulating endothelial progenitor cells as an early risk factor of subclinical atherosclerosis in systemic lupus erythematosus. Rheumatology (Oxford) 2014 Apr;53(4):631-638.
(32) Ebner P, Picard F, Richter J, Darrelmann E, Schneider M, Strauer BE, et al. Accumulation of VEGFR-2+/CD133+ cells and decreased number and impaired functionality of CD34+/VEGFR-2+ cells in patients with SLE. Rheumatology (Oxford) 2010 Jan;49(1):63-72.
(33) Ruger B, Giurea A, Wanivenhaus AH, Zehetgruber H, Hollemann D, Yanagida G, et al. Endothelial precursor cells in the synovial tissue of patients with rheumatoid arthritis and osteoarthritis. Arthritis Rheum 2004 Jul;50(7):2157-2166.
(34) Silverman MD, Haas CS, Rad AM, Arbab AS, Koch AE. The role of vascular cell adhesion molecule 1/ very late activation antigen 4 in endothelial progenitor cell recruitment to rheumatoid arthritis synovium. Arthritis Rheum 2007 Jun;56(6):1817-1826.
(35) Isozaki T, Arbab AS, Haas CS, Amin MA, Arendt MD, Koch AE, et al. Evidence That CXCL16 Is a Potent Mediator of Angiogenesis and Is Involved in Endothelial Progenitor Cell Chemotaxis : Studies in Mice With K/BxN Serum-Induced Arthritis. Arthritis Rheum 2013 Jul;65(7):1736-1746.
(36) Webster C, Blau HM. Accelerated age-related decline in replicative life-span of Duchenne muscular dystrophy myoblasts: implications for cell and gene therapy. Somat Cell Mol Genet 1990 Nov;16(6):557-565.
(37) Jodon de Villeroche V, Avouac J, Ponceau A, Ruiz B, Kahan A, Boileau C, et al. Enhanced late-outgrowth circulating endothelial progenitor cell levels in rheumatoid arthritis and correlation with disease activity. Arthritis Res Ther 2010;12(1):R27.

(38) Rodriguez-Carrio J, Prado C, de Paz B, Lopez P, Gomez J, Alperi-Lopez M, et al. Circulating endothelial cells and their progenitors in systemic lupus erythematosus and early rheumatoid arthritis patients. Rheumatology (Oxford) 2012 Oct;51(10):1775-1784.
(39) Kim HK, Song KS, Kim HO, Chung JH, Lee KR, Lee YJ, et al. Circulating numbers of endothelial progenitor cells in patients with gastric and breast cancer. Cancer Lett 2003 Jul 30;198(1):83-88.
(40) Egan CG, Lavery R, Caporali F, Fondelli C, Laghi-Pasini F, Dotta F, et al. Generalised reduction of putative endothelial progenitors and CXCR4-positive peripheral blood cells in type 2 diabetes. Diabetologia 2008 Jul;51(7):1296-1305.
(41) Xia WH, Yang Z, Xu SY, Chen L, Zhang XY, Li J, et al. Age-related decline in reendothelialization capacity of human endothelial progenitor cells is restored by shear stress. Hypertension 2012 Jun;59(6):1225-1231.

CHAPTER 3

Does reduction of disease activity improve early markers of cardiovascular disease in newly diagnosed Rheumatoid Arthritis patients?

Lodewijk de Groot, Nynke A. Jager, Johanna Westra, Andries J. Smit, Cees G.M. Kallenberg, Marcel D. Posthumus, Marc Bijl

Rheumatology (accepted)

Abstract

OBJECTIVES The incidence of cardiovascular disease (CVD) is increased in rheumatoid arthritis (RA). The current study was designed to evaluate whether reduction of disease activity will influence early markers of cardiovascular disease.

METHODS In a prospective longitudinal study 58 newly diagnosed RA patients and 58 age and sex matched healthy controls (HC) were included. Endothelial dysfunction was measured by small artery elasticity (SAE), endothelial cell activation was assessed by measuring soluble vascular cellular activation molecule-1 (sVCAM-1) and von Willebrand Factor (vWF). Advanced glycation endproducts (AGEs) were quantified by skin autofluorescence. After one year measurements were repeated in all RA patients.

RESULTS At entry, SAE was decreased in RA (median 3.4 ml/mmHg100, range 1.2-9.0) vs. HC (6.1, 5.0-15.3), P<0.0001 and sVCAM-1 and vWF were increased: 391 ng/ml (256-680) vs. 341 (223-691), P=0.0015 and 120 ng/ml (26.5-342) vs. 99 (22-298), P=0.02, respectively. SAE was inversely correlated with DAS-28 (r=-0.31, P=0.016). AGEs were increased 2.55 a.u. (1.29-4.65) vs. 2.12 (1.32-3.82) in HC P=0.003. In multivariate analysis, presence of RA, age and systolic blood pressure were independently and inversely related to SAE. After one year SAE had significantly improved in RA from 3.4 (1.2-9.0) to 3.8 (1.5-10.3), P=0.03.

CONCLUSION Endothelial dysfunction is present in newly diagnosed RA patients, independently of traditional risk factors and is inversely correlated with disease activity. By reducing disease activity endothelial dysfunction improves, though not to normal values. Besides reduction in disease activity targeting traditional risk factors remains important in preventing CVD in RA.

Keywords: Rheumatoid arthritis, endothelial cell activation, endothelial dysfunction, small artery elasticity, intima media thickness, advanced glycation end products, atherosclerosis

Introduction

Morbidity, and mortality due to cardiovascular diseases (CVD) in RA is increased (1,2). CVD is the result of atherosclerosis and formation of atherogenic plaques (3). Three stages are identified in the formation of atherogenic plaques: Endothelial cell (EC) activation, endothelial cell dysfunction and finally preclinical atherosclerosis (4). In addition to known traditional risk factors such as smoking, hypertension, overweight and dyslipidemia, other non-traditional-risk factors are supposed to play an important role in increased cardiovascular mortality and morbidity in RA. Among these, CRP, auto-antibodies, oxidized LDL and Advanced Glycation Endproducts (AGEs) are recognized (5). AGEs accumulate in tissues with slow turnover such as the vessel wall, cartilage and skin. AGEs can be quantified in the skin, urine and serum and are increased in longstanding RA (6). AGEs can ligate to the receptor of AGE (RAGE), which, as it is present on endothelial cells, results in EC activation and finally formation of sVCAM-1 (7). In this way the AGE-RAGE interaction becomes a self maintaining and re-enforcing process. We hypothesize that systemic inflammation is a major non-traditional risk factor that contributes significantly to the development of premature atherosclerosis in RA. We hypothesize that even in newly diagnosed RA AGE accumulation and early stages of atherosclerosis, i.e. endothelial activation and dysfunction, will be present and that these changes can be reversed by reducing disease activity.

Methods

Patients and controls

The study was designed as a prospective longitudinal study with RA patients compared with healthy controls at inclusion (t=0). After one year patients served as their own controls. Consecutive patients with age 18-80 yrs, fulfilling the American College of Rheumatology (ACR) criteria for RA (8) and having symptoms of RA since maximum one year, were asked to participate. The patients had to be mentally able to understand the study information and had to subscribe the informed consent. Patients with diabetes mellitus (fasting blood glucose level> 7 mmol/L, or use of antidiabetic drugs), pregnancy, renal impairment (serum creatinin> 140µmol/L) or surgery or myocardial infarction in the past three months were excluded. In total 58 patients and 58 healthy controls (HC) were included. Patients and HC were recruited between august 2008 and september 2010. Information concerning traditional risk factors for CVD was obtained from all study participants, as decribed earlier (9). Patients were treated according to the DREAM protocol, a DAS-guided scheme, aiming at remission (DAS-28<2.6) (10). Treatment for hypertension or dyslipidemia was not pre-specified in the protocol and at the discretion of the treating physician. The study was approved by the local medical ethics committee of the University Medical Center of Groningen and informed consent was obtained from all study participants.

Blood sampling and analysis

After an overnight fast blood was sampled and creatinine, total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and glucose were measured using routine procedures. Serum was stored at -20˚C for analysis of endothelial cell activation markers.

Measurement of disease activity, joint damage and cumulative CRP

For assessment of disease activity the Disease Activity Score for 28 joints was used (DAS-28) (11,12). Cumulative CRP was calculated as described (13).

Measurement of Endothelial cell activation markers

Serum sVCAM-1 levels were measured by ELISA according to manufacturers guidelines (R&D Systems, Abingdon, UK). vWF was measured by ELISA as previously described (14).

Measurement of Small Artery Elasticity (SAE), intima-media thickness (IMT), and AGEs

SAE was measured as described before (15). Coefficient of variation for SAE was 11.2% (16). IMT was measured in a standardized way by the same experienced technicians. Coefficient of variation of IMT measurement is approximately 5% (17,18). AGEs were measured using the auto-fluorescence reader (AGE-reader, DiagnOptics Technologies BV Groningen, The Netherlands). Coefficient of variance is 5.8% (19).

Statistical methods

Data are expressed as median (range), unless stated otherwise. The sample size of 60 patients was calculated on the assumption that 50% of RA patients would achieve remission, and SAE in these patients would normalize (from 4.5 to 7.7 ml/mmHg100; with SD of 3.7 found in an earlier study (20) Expecting 5% loss to follow-up 60 patients were invited. Two-sample t-tests or Mann-Whitney-U tests were used as appropriate to make comparisons between patients and controls for continuous variables. For categorical variables the chi-square method and for very small expected frequencies the Fisher's exact test were used. Gaussian distribution of the data was analyzed with D'Agostino and Pearson omnibus normality test. Correlation analysis was performed by Pearson correlation when variables were normally distributed, otherwise the Spearman correlation was used. To control for unbalanced cardiovascular risk factors between patients and HC multivariate regression analysis was used. Multivariate linear regression analysis for predictors of SAE at t=0 was performed by using forward inclusion of variables with P values ≤ 0.10 in univariate analysis. The probability of F for entry was 0.05, implicating that variables are included in the model until the statistical significance does not improve anymore. Longitudinal data were analyzed using Wilcoxon matched-pairs signed rank test. Analyses were performed using GraphPad Prism version 4.03 2005 and SPSS version 14.0. Two-sided P-values <0.05

were considered significant.

Results

Clinical characteristics of patients and controls

RA patients had an unfavorable cardiovascular risk profile. In comparison to HC, RA patients were more often smokers, had more frequently hypertension, had lower HDL-levels, and higher triglyceride levels. Most patients were on NSAIDs. Use of antihypertensive medication as well as statin use remained constant. After one year a second measurement could be obtained in all but 5 patients.

Endothelial dysfunction, endothelial activation, intima media thickness and AGEs

At inclusion, endothelial dysfunction, as reflected by SAE was present in RA patients. SAE was 3.4 ml/mmHg100 (1.2-9.0) vs. 6.1 (2.0-15.3) in HC (P<0.0001). Endothelial activation was present in patients as well. sVCAM-1 in RA patients was 391 ng/ml (256-680) vs. 341 (223-691) in HC (P=0.0015) and vWF was 120 ng/ml (26.5-342) vs. 99 (22-298) P=0.017. IMT was not different between RA and HC on t=0, being 0.73mm (0.45-1.64) in RA vs. 0.72 (0.39-1.46) in HC. AGEs were increased in RA (2.55 a.u. (1.29-4.65) vs. 2.12 (1.32-3.82) in HC P=0.003. In multivariate analysis with forward inclusion of variables that had a P value <0.10 in univariate analysis, endothelial dysfunction was independently associated with the presence of RA, age and systolic blood pressure. The adjusted R^2 of this model was 0.454 (table 1).In RA patients, SAE was inversely correlated with DAS-28, (r=-0.30, P=0.03), AGE's (r=-0.33, P=0.016), systolic blood pressure (r=-0.56, P<0.0001) and IMT (r=-0.26, P=0.05). AGEs correlated with DAS-28 (r= 0.29, P= 0.03), systolic RR (r=0.37, P=0.005), glucose levels (r=0.35, P=0.01) and IMT (r=0.55, P<0.0001)

Table 1. Univariate analysis and multivariate linear regression analysis with forward entry of variables with P<0.10 in univariate analysis on t=0 with SAE as dependent variable.

	Univariate analysis		Multivariate analysis	
	B (95% C.I.)	P-value	B (95% C.I.)	P-value
Age	0.123 (-0.162--0.083)	<0.0001	-0.095 (-0.134--0.056)	<0.0001
Male gender	-0.690 (-1.758-0.378)	0.203		
BMI	-0.033 (-0.181-0.115)	0.661		
Dyslipidemia	-0.502 (-1.561-0.557)	0.349		
Tot. chol.	-0.155 (-0.604-0.294)	0.496		
HDL-chol.	0.622 (-0.306-1.631)	0.178		
LDL-chol	-0.060 (-0.623-0.503)	0.834		
Trigl.	-0.464 (-0.1220-0.292)	0.227		
Hypertension	-2.633 (-3.598--1.668)	<0.0001		
RR syst.	-0.093 (-0.121--0.065)	<0.0001	-0.047 (-0.076--0.018)	0.002
RR diast.	-0.107 (-0.158--0.056)	<0.0001		
R.A.	-2.164 (-3.136--1.192)	<0.0001	-0.1828 (-2.641--1.015)	<0.0001
Active smoking	-1.663 (-2.875--0.451)	0.008		
Creatinin	0.015 (-0.02-0.050)	0.412		
Glucose	-0.265 (-0.659-0.128)	0.184		
IMT	-3.283 (-5.056--1.511)	<0.0001		

All data are represented as B with 95% confidence interval. The adjusted R-square of this model was 0.454.

Longitudinal data

DAS-28 improved from 4.64 (range 2.1-7.8) to 2.15 (0.17-5.53) P< 0.0001. After 1 year (t=1) SAE improved in RA patients, although it did not normalize to levels found in HC. SAE increased from 3.4, (1.2-9.0) to 3.8, (1.5-10.3), P=0.03. No significant change was found in sVCAM-1, vWF, IMT or AGEs (data not shown). Systolic blood pressure did not change significantly, whereas diastolic blood pressure decreased slightly from median 80 mmHg (56-108) to 79 (58-112), P=0.03. Total cholesterol increased from 5.0 mmol/L (2.9-9.1) to 5.5 (3.7-10.1), HDL increased from 1.2 mmol/L (0.8-2.7) to 1.5 (0.8-3.0), and LDL increased from 3.2 mmol/L (1.4-6.5) to 3.7 (1.3-6.7). Only the rise in HDL reached significance (P=0.0001). Change in DAS-28 did not correlate with the change in SAE, also no relation was found between change in SAE and cumulative CRP. To further substantiate the relation between disease activity, endothelial activation and dysfunction RA patients were divided in those reaching remission (DAS < 2.6) after one year versus those not reaching remission. Patients in remission (n=36) showed improvement in SAE (from 3.2 (1.2-9.0) to 3.9 (1.5-10.3), P=0.02)

whereas those not reaching remission (n=15) had a deterioration in SAE from 4.0 (1.3-8.0) to 3.8 (1.5-8.4) P=0.6. No difference was found in endothelial activation markers and AGEs regarding patients in remission vs. no remission (table 2).

Table 2. Comparison of patients in remission vs patients not in remission on t=1

	Remission (n=36)			No remisson (n=15)		
	t=0	**t=1**	**P-value**	**t=0**	**t=1**	**P-value**
SAE	3.2 (1.2-9.0)	3.9 (1.5-10.3)	0.02	4.0 (1.3-8.0)	3.8 (1.5-8.4)	0.61
VCAM	377 (256-680)	338 (224-637)	0.13	410(297-620)	370 (235-757)	0.63
vWF	115 (35-328)	120 (38-321)	0.28	173 (27-342)	195 (42-831)	0.50
AGEs	2.53 (1.29-3.45)	2.60 (1.52-4.29)	0.67	2.62 (1.44-4.65)	2.54 (1.85-3.57)	0.79
IMT	0.73 (0.49-1.16)	0.74 (0.48-1.21)	0.61	0.79 (0.45-1.62)	0.83 (0.50-1.58)	0.13
DAS-28	4.5 (2.1-7.8)	1.9 (0.17-2.58)	<0.0001	5.1 (2.9-7.8)	3.2 (2.6-5.5)	0.0004

SAE; Small Artery Elasticity, VCAM; Vascular Cellular Adhesion Molecule, vWF; von Willebrand Factor, AGEs; Advanced Glycation Endproducts, IMT; Intima media Thickness, DAS-28; Disease Activity Score of 28 joints

Discussion

Our study shows that already very early in the course of RA endothelial activation and dysfunction are present as well as increased accumulation of AGEs. It remains to be established whether these changes occur in the very short period (<1 year) of overt clinical disease, or reflect a much longer period of subclinical inflammation. It is known from previous studies that already years before the actual diagnosis of RA levels of inflammatory cytokines like Il-6, TNF-α and IFN-γ are increased, indicating presence of subclinical inflammation (21). The unaltered IMT, despite increased presence of several traditional cardiovascular risk factors, in our patients at inclusion, argues against longstanding pro-atherosclerotic events before disease onset. This suggests that disease activity in itself indeed contributes significantly to the development of atherosclerosis, which is supported by the inverse relation we found between SAE and DAS-28. Furthermore, in multivariate analysis presence of RA proved to be an independent risk factor for decreased SAE at baseline.

Another, cross-sectional, study performed in patient with early RA not only showed a decreased SAE in RA patients, but also a significant increase in IMT (22). The fact that this study as well as other studies (23-25) in contrast to our study did show an increase of IMT even at baseline in early RA might be due to differences in methods or patient characteristics. For example in our study median DAS-28 was 4.64, in comparison to 5.6 in the study of Adhikari et al (22).

In contrast to the unaltered IMT, we found that even in early RA at time of diagnosis AGEs

in the skin were increased, suggesting that a subclinical (inflammatory) process proceeds the occurrence of clinical RA. So far no studies have been performed in early RA showing an increase in AGEs in the skin. AGEs in the skin indicate that the body has been exposed to oxidative stress or higher glucose levels. In diabetes increase of AGEs has been shown to be a predictor of macro- and microvascular events (26,27). It may be that also in RA increase of AGEs in the skin indicates risk for future cardiovascular events, in particular because, in contrast to SAE, AGEs persisted at higher levels despite lowering disease activity.

Endothelial function improved after one year of treatment, but this improvement was relatively low and SAE by far did not reach normal values. The fact that systolic blood pressure did not decrease in RA may contribute in the failure to reach normal SAE values. Patients who received remission though showed an improvement in SAE, whereas patients who did not reach remission did not change. Together, these findings support the recommendation that reduction of disease activity might reduce progression of CVD in RA. The GO-BEFORE trial in early RA showed superiority of golimumab+MTX over MTX monotherapy in reducing markers of endothelial activation (28). Several other studies have been performed that showed a better improvement in endothelial dysfunction in established RA, especially when treated with TNF blocking agents (29-32). However these studies were performed in established RA with anti-TNF as DMARD therapy. Also disease activity was much higher in these studies. One study also showed improvement in SAE in an early RA cohort (33).

It might be that the period of one year of treatment is too short to show an improvement of the endothelial function to normal levels. Alternatively, and supported by our data, even after reaching remission endothelial activation still remains present, making normalization of SAE, as part of the same process, unlikely. The fact that AGEs are increased at baseline might be of importance in this concept. Formation of AGE's is a self reinforcing process and as such might drive the atherosclerotic process independent of clinically active disease. Our data suggest that even earlier intervention or longer follow-up is needed to address this issue.

Conclusions

Our study shows that endothelial dysfunction is present in early RA and can only very partially be improved by reducing disease activity in the first year of treatment. This indicates that reducing traditional risk factors remains important in preventing CVD in RA.

References

(1) Solomon DH, Karlson EW, Rimm EB, Cannuscio CC, Mandl LA, Manson JE, et al. Cardiovascular morbidity and mortality in women diagnosed with rheumatoid arthritis. Circulation 2003 Mar 11;107(9):1303-1307.
(2) van Halm VP, Peters MJ, Voskuyl AE, Boers M, Lems WF, Visser M, et al. Rheumatoid arthritis versus diabetes as a risk factor for cardiovascular disease: a cross-sectional study, the CARRE Investigation. Ann Rheum Dis 2009 09;68(1468-2060; 1468-2060; 9):1395-1400.
(3) Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999 01/14;340(0028-4793; 0028-4793; 2):115-126.
(4) de Groot L, Posthumus MD, Kallenberg CG, Bijl M. Risk factors and early detection of atherosclerosis in rheumatoid arthritis. Eur J Clin Invest 2010 Sep;40(9):835-842.
(5) Libby P. Role of inflammation in atherosclerosis associated with rheumatoid arthritis. Am J Med 2008 10;121(1555-7162; 1555-7162; 10):S21-S31.
(6) de Groot L, Hinkema H, Westra J, Smit AJ, Kallenberg CG, Bijl M, et al. Advanced glycation endproducts are increased in rheumatoid arthritis patients with controlled disease. Arthritis Res Ther 2011 Dec 14;13(6):R205.
(7) Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. J Biol Chem 1997 07/11;272(0021-9258; 0021-9258; 28):17810-17814.
(8) Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988 Mar;31(3):315-324.
(9) Brevetti G, Silvestro A, Schiano V, Chiariello M. Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index. Circulation 2003 Oct 28;108(17):2093-2098.
(10) Vermeer M, Kuper HH, Hoekstra M, Haagsma CJ, Posthumus MD, Brus HL, et al. Implementation of a treat-to-target strategy in very early rheumatoid arthritis: results of the Dutch Rheumatoid Arthritis Monitoring remission induction cohort study. Arthritis Rheum 2011 Oct;63(10):2865-2872.
(11) Prevoo ML, 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995 01;38(0004-3591; 0004-3591; 1):44-48.
(12) van der Heijde DM, 't Hof MA, van Riel PL, Theunisse LA, Lubberts EW, van Leeuwen MA, et al. Judging disease activity in clinical practice in rheumatoid arthritis: first step in the development of a disease activity score. Ann Rheum Dis 1990 11;49(0003-4967; 0003-4967; 11):916-920.
(13) Crilly MA, Kumar V, Clark HJ, Scott NW, Macdonald AG, Williams DJ. Arterial stiffness and cumulative inflammatory burden in rheumatoid arthritis: a dose-response relationship independent of established cardiovascular risk factors. Rheumatology (Oxford) 2009 12;48(1462-0332; 1462-0332; 12):1606-1612.
(14) de Leeuw K, Nienhuis H, Smit A, Stegeman C, Kallenberg C, Bijl M. Increased accumulation of advanced glycation endproducts in patients with Wegener's granulomatosis. Ann Rheum Dis 2010 03;69(1468-2060; 0003-4967; 3):625-627.

(15) Nienhuis HL, de Leeuw K, Bijzet J, Smit A, Schalkwijk CG, Graaff R, et al. Skin autofluorescence is increased in systemic lupus erythematosus but is not reflected by elevated plasma levels of advanced glycation endproducts. Rheumatology (Oxford) 2008 Oct;47(10):1554-1558.
(16) Nienhuis HL, de Leeuw K, Bijzet J, van Doormaal JJ, van Roon AM, Smit AJ, et al. Small artery elasticity is decreased in patients with systemic lupus erythematosus without increased intima media thickness. Arthritis Res Ther 2010;12(5):R181.
(17) de Groot E, Zwinderman AH, van der Steen AF, Ackerstaff RG, Montauban van Swijndregt AD, Bom N, et al. Variance components analysis of carotid and femoral intima-media thickness measurements. REGRESS Study Group, Interuniversity Cardiology Institute of The Netherlands, Utrecht, The Netherlands. Regression Growth Evaluation Statin Study. Ultrasound Med Biol 1998 07;24(0301-5629; 0301-5629; 6):825-832.
(18) Sramek A, Bosch JG, Reiber JH, Van Oostayen JA, Rosendaal FR. Ultrasound assessment of atherosclerotic vessel wall changes: reproducibility of intima-media thickness measurements in carotid and femoral arteries. Invest Radiol 2000 Dec;35(12):699-706.
(19) Meerwaldt R, Graaff R, Oomen PH, Links TP, Jager JJ, Alderson NL, et al. Simple non-invasive assessment of advanced glycation endproduct accumulation. Diabetologia 2004 07;47(0012-186; 7):1324-1330.
(20) Van Doornum S, McColl G, Jenkins A, Green DJ, Wicks IP. Screening for atherosclerosis in patients with rheumatoid arthritis: comparison of two in vivo tests of vascular function. Arthritis Rheum 2003 01;48(0004-3591; 1):72-80.
(21) Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLoS One 2012;7(5):e35296.
(22) Chatterjee Adhikari M, Guin A, Chakraborty S, Sinhamahapatra P, Ghosh A. Subclinical atherosclerosis and endothelial dysfunction in patients with early rheumatoid arthritis as evidenced by measurement of carotid intima-media thickness and flow-mediated vasodilatation: an observational study. Semin Arthritis Rheum 2012 Apr;41(5):669-675.
(23) Hannawi S, Marwick TH, Thomas R. Inflammation predicts accelerated brachial arterial wall changes in patients with recent-onset rheumatoid arthritis. Arthritis Res Ther 2009;11(1478-6362; 1478-6362; 2):R51.
(24) Hannawi S, Haluska B, Marwick TH, Thomas R. Atherosclerotic disease is increased in recent-onset rheumatoid arthritis: a critical role for inflammation. Arthritis Res Ther 2007;9(1478-6362; 6):R116.
(25) Georgiadis AN, Voulgari PV, Argyropoulou MI, Alamanos Y, Elisaf M, Tselepis AD, et al. Early Treatment Reduces the Cardiovascular Risk Factors in Newly Diagnosed Rheumatoid Arthritis Patients. Semin Arthritis Rheum 2008 01/11(0049-0172).
(26) Goh SY, Cooper ME. Clinical review: The role of advanced glycation end products in progression and complications of diabetes. J Clin Endocrinol Metab 2008 04;93(0021-972; 0021-972; 4):1143-1152.
(27) Gerrits EG, Lutgers HL, Kleefstra N, Graaff R, Groenier KH, Smit AJ, et al. Skin autofluorescence: a tool to identify type 2 diabetic patients at risk for developing microvascular complications. Diabetes Care 2008 03;31(1935-5548; 1935-5548; 3):517-521.
(28) Kirkham BW, Wasko MC, Hsia EC, Fleischmann RM, Genovese MC, Matteson EL, et al. Effects of golimumab, an anti-tumour necrosis factor-alpha human monoclonal antibody, on lipids and markers of inflammation. Ann Rheum Dis 2014 Jan;73(1):161-169.
(29) Hurlimann D, Forster A, Noll G, Enseleit F, Chenevard R, Distler O, et al. Anti-tumor necrosis factor-alpha treatment improves endothelial function in patients with rheumatoid arthritis. Circulation 2002 10/22;106(1524-4539; 1524-4539; 17):2184-2187.

(30) Wong M, Oakley SP, Young L, Jiang BY, Wierzbicki A, Panayi G, et al. Infliximab improves vascular stiffness in patients with rheumatoid arthritis. Ann Rheum Dis 2009 08;68(1468-2060; 1468-2060; 8):1277-1284.
(31) Maki-Petaja KM, Hall FC, Booth AD, Wallace SM, Yasmin, Bearcroft PW, et al. Rheumatoid arthritis is associated with increased aortic pulse-wave velocity, which is reduced by anti-tumor necrosis factor-alpha therapy. Circulation 2006 09/12;114(1524-4539; 11):1185-1192.
(32) Maki-Petaja KM, Elkhawad M, Cheriyan J, Joshi FR, Ostor AJ, Hall FC, et al. Anti-tumor necrosis factor-alpha therapy reduces aortic inflammation and stiffness in patients with rheumatoid arthritis. Circulation 2012 Nov 20;126(21):2473-2480.
(33) Guin A, Chatterjee Adhikari M, Chakraborty S, Sinhamahapatra P, Ghosh A. Effects of disease modifying anti-rheumatic drugs on subclinical atherosclerosis and endothelial dysfunction which has been detected in early rheumatoid arthritis: 1-year follow-up study. Semin Arthritis Rheum 2013 Aug;43(1):48-54.

CHAPTER 4

Skin autofluorescence – a marker for advanced glycation end products – is positively associated with plasma levels of matrix metalloproteinase in subjects without overt atherosclerosis.

Nynke A. Jager, Helmy J. Hinkema, Joop D. Lefrandt, Clark J. Zeebregts, Damiano Baldassare, Andries J. Smit, Douwe J. Mulder, Johanna Westra.

Submitted for publication

Abstract

OBJECTIVES Advanced glycation end products (AGEs) have been shown to influence the in vitro production of matrix metalloproteinases (MMPs), thereby propagating atherosclerosis development. We tested the association between skin auto fluorescence (skin AF, a non-invasive technique to measure tissue AGEs), inflammation (hsCRP), sRAGE and plasma levels of MMPs as well as different stages of atherosclerotic plaque development (measured by IMT) in healthy individuals.

METHODS In 262 healthy subjects with a Framingham Risk score ranging form 0 to 15%, skin AF, mean and maximum intima media thickness (IMT) of the common carotid artery was measured. Subjects were divided in three groups; subjects without atherosclerosis (plaque score 0), intermediate atherosclerosis (plaque in 1-3 segments) and atherosclerosis in more than 4 segments (high, plaque score ≥ 4). Furthermore, plasma levels of sRAGE and hsCRP were measured. Also serum levels of MMPs (MMP-3, MMP-9) and tissue inhibitor of metalloproteinase- 1 (TIMP- 1)) were determined.

RESULTS Skin AF correlated with MMP-3 ($r=0.26$, $P<0.0001$), MMP-9 ($r=0.31$, $P<0.0001$), TIMP-1 ($r=0.25$, $P<0.0001$) and hsCRP ($r=0.26$, $P<0.0001$) in univariate analyses. In contrast, no correlation between skin AF and sRAGE could be found. Furthermore, skin AF correlated with mean IMT ($r=0.35$, $P<0.0001$) and maximal IMT ($r=0.17$, $P=0.008$), and FRS scores were related to skin AF. Skin AF and biomarkers were significantly higher in subjects with atherosclerotic plaque (intermediate, score of 1-3), compared to subjects without atherosclerosis in the carotid arteries. MMP-9 and hsCRP were significantly lower in the intermediate plaque group compared to the highest group.

CONCLUSION Skin AF is positively associated with MMPs and TIMP-1 in plasma and with FRS. MMP-9 had the best correlation with skin AF in univariate analysis, and was significantly different in all three plaque groups. Skin AF together with plasma MMP-9 might be a good indicator for cardiovascular risk in subjects without overt atherosclerosis.

Introduction

Atherosclerosis is an inflammatory disease mediated by cellular mechanisms driven by oxidative stress (1). Advanced glycation end products (AGEs) are stable structures accumulating on long-lived proteins, formed by non-enzymatic glycation and oxidative reactions (2). AGEs are implicated in long term complications of diabetes mellitus and renal disease, and evidence for an important role in cardiovascular disease beyond these conditions is growing (3). By interaction with their major receptor (RAGE), AGEs have been shown *in vitro* to promote inflammatory mechanisms leading to atherosclerotic plaque formation and vulnerability (4). However, little clinical evidence is available to substantiate these observations.

Matrix metalloproteinases (MMPs) play an important role in virtually all stages of atherosclerosis development (5). Already in early stages of plaque formation, MMP production by inflammatory cells induces basal membrane degradation, promoting smooth muscle cell migration and plaque development. Furthermore, extensive literature has demonstrated that MMP activity, especially in the shoulders of plaques, leads to instability ensuing acute cardiovascular events including ischemic stroke and acute coronary syndromes (6). *In vitro* (7) and *ex vivo* (8,9), evidence is growing that AGE production is linked to the MMP metabolism. AGEs co-localize in intimal areas of both aortic and coronary specimens and by engagement with RAGE MMP expression on inflammatory cells is induced (10). These events contribute to plaque destabilization by inducing culprit MMP expression and may potentially play an important role in atherosclerotic plaque development and instability.

In clinical studies, measurement of AGEs in plasma is difficult. We developed and extensively validated a non-invasive technique to measure tissue AGEs by evaluating skin autofluorescence (skin AF) (11). Skin AF is indeed a strong predictor of cardiovascular (CV) mortality (12). Furthermore, earlier studies have indicated that skin AF is elevated in coronary artery disease, irrespective of diabetes mellitus (DM) or renal disease (13), correlates with carotid intima media thickness (IMT) (14), and is elevated in patients with carotid artery stenosis and peripheral artery disease (PAD) (15). Furthermore, skin AF was shown to be associated with CRP in patients with several conditions, including central obesity (16), DM (17), hemodialysis (18) and coronary artery disease (19), and correlated with the soluble isotype of RAGE (sRAGE) in coronary artery disease (19) and inversely related to HDL anti-oxidative capacity in DM (20).

Taking the above mentioned evidence into account, we believe that AGEs might play an important role in the inflammatory process leading to atherosclerosis development. The present study was initiated to test whether plasma levels of MMP-3, -9, TIMP-1 and hsCRP and sRAGE, as well as atherosclerotic plaque development (measured by IMT), are associated with tissue levels of AGEs (measured by skin AF) in subjects without cardiovascular disease (CVD), but with a wide range in Framingham Risk Scores (FRS).

Materials and Methods

Study design

This study involved 262 healthy subjects of at least 18 years of age. To obtain a wide range of FRS in subjects without a history of CVD, subjects were recruited from two cohorts, i.e. a healthy group of subjects recruited by advertisement with no more than one cardiovascular risk factor (RF) and a second healthy group of subjects that participated in an international cohort, referred to as the IMPROVE study (21) who were selected on the presence of at least three RFs (dyslipidemia, hypertension, DM, smoking, or family history of cardiovascular disease). We measured skin AF, performed carotid ultrasound examinations for assessment of intima media thickness of the carotid artery and measured plasma levels of MMP-3, -9 and TIMP-1, sRAGE, and hsCRP. All measurements were compared to skin AF. Participants had to be asymptomatic for cardiovascular diseases and free of any conditions that might limit IMT visualization. Patients with a life expectancy ≤ 3 years were excluded from participation (in accordance with IMPROVE guidelines (21)). Furthermore, subjects with an estimated glomerular filtration rate of <60 ml/min/1.73 cm^2 or a history of renal transplantation, recent acute coronary syndrome, cerebrovascular attack or sepsis (all within the past 3 months), and current cancer or autoimmune disease were excluded because these conditions have previously been shown to increase skin AF. Furthermore, participants with a brown or black skin type (Fitzpatrick skin type V and VI) (22) had to be excluded as skin AF could not be measured reliably due to excessive light absorption in the skin. The study was approved by the local institutional review board and all participating subjects gave written informed consent.

Risk factor definitions

The following RFs were assessed (as formulated in the original IMPROVE study protocol): smoking status, dyslipidaemia (low density lipoprotein (LDL) cholesterol >4.0 mmol/l, high density lipoprotein (HDL) cholesterol <1.2 mmol/l (female) or HDL-cholesterol <1.0 mmol/l (male), triglycerides > 4.0 mmol/l, or current lipid lowering treatment, hypertension (≥140/90 mmHg or drug treatment for hypertension), obesity (body mass index (BMI) ≥30 kg/m^2), and DM (known diabetes or fasting plasma glucose >7.0 mmol/l or random plasma glucose >11.1 mmol/l). The Framingham risk score was defined as the 10 year risk on coronary heart disease (i.e. myocardial infarction or death from coronary heart disease) (23). Kidney function was estimated using the MDRD ('modification of diet in renal disease') formula.

Biochemical analyses

Blood was collected after overnight fasting. Venous blood samples were collected into EDTA-containing tubes (1.5 mg /mL). Lipid concentrations (total, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol and triglycerides), glucose and

creatinine were measured by routine techniques from fresh plasma. Additional plasma samples were kept at –80°C prior to analysis at the lab. HsCRP was determined by nephelometry with a lower limit of 0.175 mg/L (BNII N; Dade Behring, Marburg, Germany). Serum levels of sRAGE (R&D Systems, Minneapolis, USA) and MMP-3 (Biosource, Nivelles, Belgium) were measured using commercially available enzyme linked immunosorbent (ELISA) techniques, according to manufacturer's instructions. MMP-9 and TIMP-1 were determined as described before (24). All methods had an inter- and intra-assay variation of less than 5%.

Carotid artery atherosclerosis assessment using ultrasonographic variables

Carotid ultrasonography was performed using two closely related methods, as described earlier (21,25). Briefly, the far walls of the left and right common carotids, bifurcations, and internal carotids were visualized in anterior, lateral, and posterior projections and recorded on sVHS videotapes. All carotid measurements were averaged to calculate the mean IMT for each subject; the highest IMT value among the three carotid segments was defined as the maximal IMT. Repeatability of carotid IMT measurements was previously described (21). Presence of plaque was based on the Mannheim consensus statement (26), where a plaque is a focal structure that encroaches onto the arterial lumen at least 0.5 mm or 50% of the surrounding IMT value or demonstrates a thickness of ≥ 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface. A total plaque score was calculated summing all segments with presence of at least one plaque, consequently ranging from 0 to 6. Subjects where divided in three groups; subjects without atherosclerosis (plaque score 0, n= 64), intermediate atherosclerosis (plaque in 1-3 segments, n= 166) and atherosclerosis in more than 4 segments (high, plaque score ≥ 4, n= 32). The sonographers were unaware of the characteristics and biomarker levels of the studied persons. The measurements were analyzed offline by an independent image analyst who was unaware of the clinical status of the patient.

Measurement of skin autofluorescence

Skin AF was assessed with the AGE Reader (DiagnOptics Technologies BV, Groningen, the Netherlands). This is a non-invasive desk-top device that uses the characteristic fluorescent properties of certain AGEs to estimate the level of AGEs accumulation in the skin by illuminating a skin surface of 4 cm^2. The method has been previously validated to skin biopsy dermal tissue homogenates (27). Technical details concerning the optical technique have been extensively described elsewhere (28). Skin AF is calculated from three consecutive measurements of the right forearm as the ratio between the emission light and reflected excitation light, multiplied by 100 and expressed in arbitrary units (AU). Earlier, skin AF was shown to have an intra-individual Blant-Altman error of 5.0% on a single day, and 5.9% for seasonal variation (11).

Statistical analysis

Medians with interquartile range for nonparametric distributions and mean with standard deviation for Gaussian populations were provided. The association between skin AF and plasma biomarkers and IMT was calculated using the Pearson or Spearman correlation when appropriate. Also an ordinal logistic regression (univariate and multivariate) analysis using stepwise selection (inclusion criterium $P<0.01$) method to examine the relationship and significance between skin AF, MMP-3 and -9, TIMP-1 and hsCRP additional to classical risk factors associated to skin AF (age, diabetic status, smoking status and BMI) was done. All statistical analyses were performed using PASW Statistics version 22 (SPSS Inc, Chicago, Illinois, USA). All statistical tests were two-sided. A P-value of less than 0.05 was considered statistically significant.

Results

Subject characteristics

A total of 124 men and 138 women with a median age of 60.2 (19-81.5) years were included. The clinical and laboratory characteristics of the study population are given in Table 1. A significant correlation of skin AF with several characteristics was observed, including age (r=0.36, P<0.0001), obesity (P=0.0004), BMI (r=0.35, P<0.0001), presence of DM (P<0.001) and plasma glucose levels (r=0.26, P<0.0001). Skin AF was negatively correlated with HDLC levels (r=-0.23, P=0.0002).

Table 1. Baseline characteristics and risk factors for atherosclerosis

	Subjects (n=269)
Men, n (%)	128 (48)
Age, years	60.2 (19-81.5)
BMI, kg/m2	27.8 ± 5.2
Obesity, n (%)	75 (32)
Smoking status, n (%)	41 (15)
Past, n (%)	163 (61)
Hypertension, n (%)	185 (69)
Systolic blood pressure, mmHg	146 ± 20
Diastolic blood pressure, mmHg	83 ± 10
Hyperlipidemia, n (%)	175 (65)
Triglycerides (mmol/L)	1.3 (0.5-5.6)
Cholesterol (mmol/L)	5.3 ± 1.1
HDL (mmol/L)	1.3 ± 0.4
LDL (mmol/L)	3.3 ± 1.0
Diabetes mellitus, n (%)	33 (12)
Blood glucose (mmol/L)	4.8 (2.7-13.8)
Medication	
Statin use, n (%)	74 (28)
ACEi/ARB use, n (%)	52 (19)
Other antyhypertensive medication	150 (56)
Aspirin use	14 (5)
Antidiabetic use	21 (8)
Insulin use	5 (2)

Data are expressed as mean ± standard deviation or median (range) when appropriate.
Percentages between brackets. ACEi = angiotensin-converting enzyme inhibitor;
ARB = angiotensin receptor blocker

Skin autofluorescence (AGEs) vs plasma levels of MMPs, TIMP, hsCRP, and sRAGE

MMP-3 and -9 levels, hsCRP and TIMP-1 were positively correlated to skin AF (figure 1). No correlations were found for skin AF compared to plasma sRAGE levels.

Figure 1.

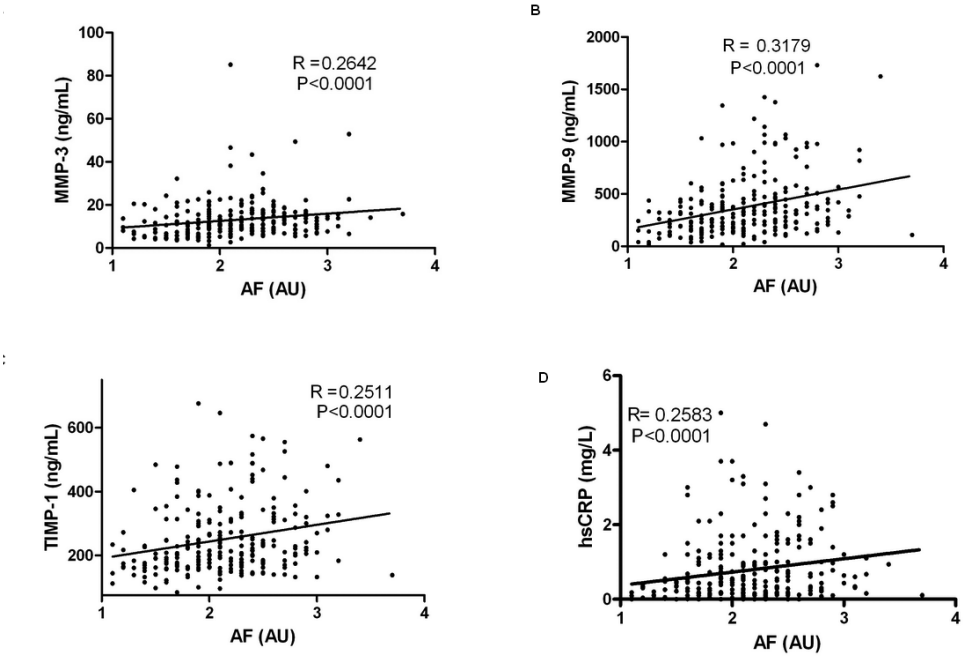


Figure 1. Skin AF was positively correlated to plasma levels of (A) MMP-3 (B) MMP-9 (C) TIMP-1 and (D) hsCRP

Univariate analyses showed plasma levels of MMP-3, -9, TIMP-1, and hsCRP were significantly associated with skin AF (P<0.001 for each). The same yields for the risk factors: BMI, DM, age, and smoking. Also MMP-3 and MMP-9 correlated to TIMP-1 (r=0.37, P<0.001 and r=0.41, P<0.001 respectively), and MMP-3 and MMP-9 correlated to each other (r=0.19, P=0.003). Multivariate linear regression analysis separately for MMP-3, MMP-9, TIMP-1 and hsCRP, together with the risk factors DM, age, BMI and smoking, showed significant association with skin AF for MMP-3 (P<0.001), MMP-9 (P=0.05) and TIMP-1 (P=0.05, Table 2). HsCRP was not independently associated with skin AF in this model (P=0.544).

Table 2. Regression analysis of MMPs and TIMP-1 associated with skin AF, independently of age, DM, BMI, smoking and hsCRP

Univariate	B	95%CI		Beta	P
MMP-3 (ng/mL)	0.007	0.004	0.009	0.328	<0.001
MMP-9 (ng/mL)	0.005	0.003	0.006	0.336	<0.001
TIMP-1 (ng/mL)	0.009	0.005	0.012	0.313	<0.001
Multivariate					
MMP-3 (ng/mL)*	0.004	0.002	0.006	0.200	<0.001
MMP-9 (ng/mL)*	0.002	0.000	0.004	0.125	0.050
TIMP-1 (ng/mL)*	0.003	0.000	0.007	0.118	0.049
DM (yes/no)	0.002	0.001	0.004	0.160	0.003
Age (years)	0.006	0.004	0.008	0.355	<0.001
BMI (kg/m^2)	<0.001	0.000	0.000	0.290	<0.001
Smoking (yes/no)	0.001	0.000	0.003	0.086	0.107
hsCRP (mg/mL)	<0.001	-0.002	0.001	-0.052	0.409

Univariate and multiple regression analysis using stepwise selection method. * MMP-3, -9 and TIMP-1 were analyzed separately, as they were closely related. B indicates the unstandardized coefficients with 95%-confidence interval (95%CI). Beta indicates standardized coefficient. BMI: body mass index, MMP-3: matrix metalloptroteinase-3. MMP-3 and hsCRP were log transformed, natural logarithm of age was used

Skin AF and biomarkers versus IMT measurement and plaque presence

Mean IMT (figure 2A) as well as maximal IMT (highest IMT value among the three carotid segments, figure 2B) correlated significantly with skin AF. All biomarkers were associated to mean IMT (P<0.001 for MMP-3, MMP-9 and TIMP-1, P=0.01 for hsCRP). Also, max IMT was correlated to MMP-9 and TIMP-1 (P=0.02 for both). No correlation could be found between max IMT and MMP-3 and hsCRP. Also the 10 year risk on myocardial infarction or death from coronary heart disease was correlated to skin AF, measured using the Framingham risk score (shown in figure 2C).

Figure 2.

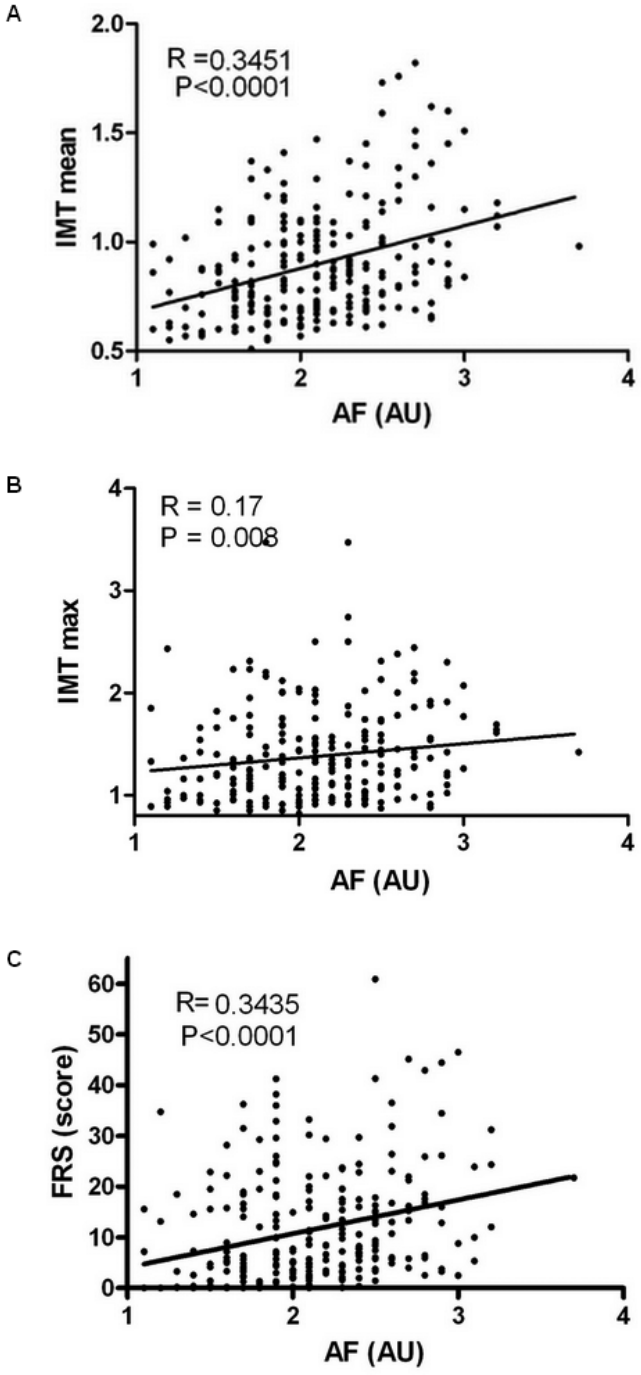


Figure 2. Skin AF was correlated to (A) mean intima media thickness and, (B) max intima media thickness. The 10 year risk of cardiovascular disease using the Framingham Risk Score (FRS) is positively correlated to skin AF.

Figure 3A shows skin AF is significantly higher in subjects with atherosclerotic plaque (intermediate, score of 1-3), compared to subjects without atherosclerosis in the carotid arteries (score of 0, P=0.004). No differences were found between the intermediate group and the group with atherosclerosis in ≥ 4 segments. In the intermediate plaque group, MMP-3, MMP-9, TIMP-1 and hsCRP levels were significantly higher compared to subjects without atherosclerosis in the carotid arteries (score of 0, P<0.001 for all). MMP-9 and hsCRP were significantly lower in the intermediate plaque group compared to the highest group (P=0.04 and P=0.01 respectively, shown in figure 3B and C). No differences were found in MMP-3 and TIMP-1 between these groups.

Figure 3.

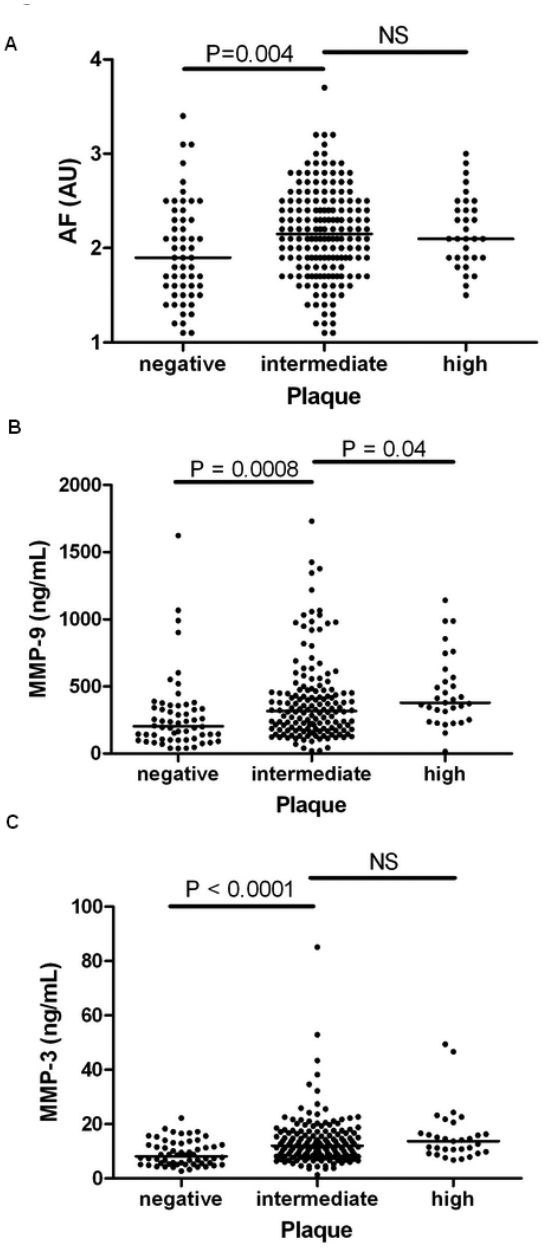


Figure 3. (A) Skin AF is higher in subjects with intermediate plaque (atherosclerosis in 1-3 segments) compared to subjects without plaque (0 segments). No difference was found between the intermediate and high group (≥ 4 segments) (B) MMP-9 is higher in subjects with intermediate plaque compared to subjects without plaque (P=0.0008), and higher in the high group compared to the intermediate group (P=0.04). (C) hsCRP is higher in subjects with intermediate plaque compared to subjects without plaque (P=0.0001), and higher in the high group compared to the intermediate group (P=0.01).

Discussion

The current study demonstrates that skin AF, which is a non-invasive measurement for tissue AGEs, is positively associated with plasma levels of MMP-3, -9, TIMP-1, hsCRP, Framingham risk score and IMT in subjects without cardiovascular disease. These data suggests the importance of AGEs in atherosclerotic plaque formation and potentially plaque vulnerability, as MMPs trigger plaque rupture. MMP-3, MMP-9 and TIMP-1 were associated with skin AF independent of known confounders, including age, BMI, smoking and DM, but more importantly independent of CRP as a marker for inflammation. Now, examination of carotid arteries for intima-media thickness (IMT) is a widely used and non-invasive marker to reveal the severity of atherosclerosis and evaluation of vascular stenosis. However, this conventional diagnostic imaging method is not sufficient; as the chance for an atherosclerotic plaque to cause clinical symptoms is equal to its probability to become vulnerable and rupture. Factors that promote plaque vulnerability such as presence of lipid pools and necrotic core with an overlying thin fibrous cap, increased macrophage activity, production of MMPs and plaque progression, are observed within plaques that are modest in terms of angiographic stenosis severity within positively remodeled arterial segments (29).

Based on previous research, MMP-9 seems to play the most important role in atherosclerosis. In a study by Loftus *et al.* patients undergoing carotid endarterectomy were investigated on the character, level, and expression of MMPs in carotid plaques. This was correlated to their clinical status. It was found that MMP-9 concentration was significantly higher in symptomatic patients compared to asymptomatic patients. No difference in the levels of MMP-1, -2, or -3 was found between symptomatic and asymptomatic patients (30). Furthermore, another study reported segments at or near the bifurcation and segments with intraplaque hemorrhage contained higher MMP-9 levels and activity compared to segments distant from the bifurcation. TIMPs were highly abundant in fibrotic and necrotic segments. Histologically stable plaques contained lesser amounts of MMPs, which were predominantly MMP-2 (31). sRAGE was not correlated to skin AF. A unified formula that takes AGEs, sRAGE, and esRAGE (endogenous secretary RAGE) into consideration might be a better biomarker than sRAGE alone for all AGE-RAGE-associated diseases, as these biomarkers react dependently (32).

To the best of our knowledge, this is the first study to demonstrate the association between non-invasively assessed tissue AGEs and plasma levels of MMP-3, 9 and TIMP-1 *in vivo*. However, plasma levels of AGEs have been shown to correlate with plasma levels of MMP-9 in patients with myocardial infarction before (33). Also a study by Ishibashi *et al*, showed MMP-9 expression was correlated to AGE accumulation histologically in autopsy specimens from the aortae and coronary arteries (34), and Cipollone *et al.* showed atherosclerotic plaques from diabetic patients had higer RAGE and MMP-9 expression found by immunohistochemistry (35). These and our results are in line with previous

studies demonstrating a relation between skin AF with inflammation, in autoimmune diseases which are strongly inflammation driven. In systemic sclerosis, skin AF correlated with CRP (36)and in rheumatoid arthritis skin AF correlated with vWF and sVCAM-1 (37). More importantly, skin AF correlated with CRP in diseases accompanied by low-grade inflammation, including in central obesity (16), Type 1 DM (17), and in hemodialysis (HD) patients (18). Furthermore, skin AF was inversely related to HDL anti-oxidative capacity in type 2 DM (20).

These data add more evidence to earlier published research, demonstrating a strong relation between skin AF and atherosclerosis. Skin AF is increased in and a strong predictor of mortality in DM and end stage renal disease (38) but it is also elevated in patients with ST-elevated myocardial infarction (39). In the latter, skin AF was also prospectively associated with major adverse cardiac events, demonstrating a potentially etiological role of the AGE burden as measured by skin AF in atherosclerosis progression. This study shows a relation between skin AF and MMPs, which play an important role in the development of a vulnerable atherosclerotic plaque.

Matrix metalloproteinases play an important role in virtually all stages of atherosclerosis development, and are related to AGEs. AGEs upregulate NF-κB, promoting inflammatory cascades, leading to CRP and MMP production. In vitro AGEs were shown to promote the expression of MMPs on monocytes (40) and human glomerular mesangial cells (41). This leads to pathological tissue damage, such as retinal damage in diabetic retinopathy (42), a process that could be reversed by an MMP inhibitor (43).

In conclusion, plasma levels of MMP-3, -9, TIMP-1 and hsCRP were correlated to skin AF. The biomarkers named above and skin AF were both associated with plaque score and IMT in subjects with a variable CVD risk profile. MMP-9 and hsCRP were significantly different in all three plaque groups. Based on previous research, MMP-9 seems to play the most important role in atherosclerosis. This might indicate that skin AF together with plasma MMP-9 is a good clinical biomarker for subclinical atherosclerosis in risk groups such as DM or arthritis patients.

Acknowledgements

We would like to thank the IMPROVE study team for their assistance in this study.

Conflict of interest statement

Dr. Smit is founder and stockholder of DiagnOptics Technologies B.V., The Netherlands, which is the manufacturer of the AGE Reader. All other authors have no conflicts of interest to declare.

References

(1) Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011 May 19;473(7347):317-325.
(2) Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. Circulation 2006 Aug 8;114(6):597-605.
(3) Del Turco S, Basta G. An update on advanced glycation endproducts and atherosclerosis. Biofactors 2012 Jul-Aug;38(4):266-274.
(4) Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, et al. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med (Berl) 2005 Nov;83(11):876-886.
(5) Chen Q, Jin M, Yang F, Zhu J, Xiao Q, Zhang L. Matrix metalloproteinases: inflammatory regulators of cell behaviors in vascular formation and remodeling. Mediators Inflamm 2013;2013:928315.
(6) Back M, Ketelhuth DF, Agewall S. Matrix metalloproteinases in atherothrombosis. Prog Cardiovasc Dis 2010 Mar-Apr;52(5):410-428.
(7) Rittie L, Berton A, Monboisse JC, Hornebeck W, Gillery P. Decreased contraction of glycated collagen lattices coincides with impaired matrix metalloproteinase production. Biochem Biophys Res Commun 1999 Oct 22;264(2):488-492.
(8) Hanssen NM, Wouters K, Huijberts MS, Gijbels MJ, Sluimer JC, Scheijen JL, et al. Higher levels of advanced glycation endproducts in human carotid atherosclerotic plaques are associated with a rupture-prone phenotype. Eur Heart J 2014 May;35(17):1137-1146.
(9) Furfaro AL, Sanguineti R, Storace D, Monacelli F, Puzzo A, Pronzato MA, et al. Metalloproteinases and advanced glycation end products: coupled navigation in atherosclerotic plaque pathophysiology? Exp Clin Endocrinol Diabetes 2012 Nov;120(10):586-590.
(10) Zhang F, Banker G, Liu X, Suwanabol PA, Lengfeld J, Yamanouchi D, et al. The novel function of advanced glycation end products in regulation of MMP-9 production. J Surg Res 2011 Dec;171(2):871-876.
(11) Meerwaldt R, Graaff R, Oomen PH, Links TP, Jager JJ, Alderson NL, et al. Simple non-invasive assessment of advanced glycation endproduct accumulation. Diabetologia 2004 Jul;47(7):1324-1330.
(12) de Vos LC, Mulder DJ, Smit AJ, Dullaart RP, Kleefstra N, Lijfering WM, et al. Skin autofluorescence is associated with 5-year mortality and cardiovascular events in patients with peripheral artery disease. Arterioscler Thromb Vasc Biol 2014 Apr;34(4):933-938.
(13) Mulder DJ, van Haelst PL, Graaff R, Gans RO, Zijlstra F, Smit AJ. Skin autofluorescence is elevated in acute myocardial infarction and is associated with the one-year incidence of major adverse cardiac events. Neth Heart J 2009 Apr;17(4):162-168.
(14) Lutgers HL, Graaff R, de Vries R, Smit AJ, Dullaart RP. Carotid artery intima media thickness associates with skin autofluoresence in non-diabetic subjects without clinically manifest cardiovascular disease. Eur J Clin Invest 2010 Sep;40(9):812-817.
(15) Noordzij MJ, Lefrandt JD, Loeffen EA, Saleem BR, Meerwaldt R, Lutgers HL, et al. Skin autofluorescence is increased in patients with carotid artery stenosis and peripheral artery disease. Int J Cardiovasc Imaging 2012 Feb;28(2):431-438.
(16) den Engelsen C, van den Donk M, Gorter KJ, Salome PL, Rutten GE. Advanced glycation end products measured by skin autofluorescence in a population with central obesity. Dermatoendocrinol 2012 Jan 1;4(1):33-38.
(17) Januszewski AS, Sachithanandan N, Karschimkus C, O'Neal DN, Yeung CK, Alkatib N, et al. Non-invasive measures of tissue autofluorescence are increased in Type 1 diabetes complications and correlate with a non-invasive measure of vascular dysfunction. Diabet Med 2012 Jun;29(6):726-733.

(18) Nagano M, Fukami K, Yamagishi S, Sakai K, Kaida Y, Matsumoto T, et al. Tissue level of advanced glycation end products is an independent determinant of high-sensitivity C-reactive protein levels in haemodialysis patients. Nephrology (Carlton) 2011 Mar;16(3):299-303.
(19) Mulder DJ, van Haelst PL, Gross S, de Leeuw K, Bijzet J, Graaff R, et al. Skin autofluorescence is elevated in patients with stable coronary artery disease and is associated with serum levels of neopterin and the soluble receptor for advanced glycation end products. Atherosclerosis 2008 Mar;197(1):217-223.
(20) Mulder DJ, de Boer JF, Graaff R, de Vries R, Annema W, Lefrandt JD, et al. Skin autofluorescence is inversely related to HDL anti-oxidative capacity in type 2 diabetes mellitus. Atherosclerosis 2011 Sep;218(1):102-106.
(21) Baldassarre D, Nyyssonen K, Rauramaa R, de Faire U, Hamsten A, Smit AJ, et al. Cross-sectional analysis of baseline data to identify the major determinants of carotid intima-media thickness in a European population: the IMPROVE study. Eur Heart J 2010 Mar;31(5):614-622.
(22) Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol 1988 Jun;124(6):869-871.
(23) Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. Circulation 1998 May 12;97(18):1837-1847.
(24) de Leeuw K, Sanders JS, Stegeman C, Smit A, Kallenberg CG, Bijl M. Accelerated atherosclerosis in patients with Wegener's granulomatosis. Ann Rheum Dis 2005 May;64(5):753-759.
(25) Terpstra WF, May JF, Smit AJ, Graeff PA, Meyboom-de Jong B, Crijns HJ. Effects of amlodipine and lisinopril on intima-media thickness in previously untreated, elderly hypertensive patients (the ELVERA trial). J Hypertens 2004 Jul;22(7):1309-1316.
(26) Touboul PJ, Hennerici MG, Meairs S, Adams H, Amarenco P, Bornstein N, et al. Mannheim carotid intima-media thickness and plaque consensus (2004-2006-2011). An update on behalf of the advisory board of the 3rd, 4th and 5th watching the risk symposia, at the 13th, 15th and 20th European Stroke Conferences, Mannheim, Germany, 2004, Brussels, Belgium, 2006, and Hamburg, Germany, 2011. Cerebrovasc Dis 2012;34(4):290-296.
(27) Smit AJ, Gerrits EG. Skin autofluorescence as a measure of advanced glycation endproduct deposition: a novel risk marker in chronic kidney disease. Curr Opin Nephrol Hypertens 2010 Nov;19(6):527-533.
(28) Mulder DJ, Water TV, Lutgers HL, Graaff R, Gans RO, Zijlstra F, et al. Skin autofluorescence, a novel marker for glycemic and oxidative stress-derived advanced glycation endproducts: an overview of current clinical studies, evidence, and limitations. Diabetes Technol Ther 2006 Oct;8(5):523-535.
(29) Puri R, Worthley MI, Nicholls SJ. Intravascular imaging of vulnerable coronary plaque: current and future concepts. Nat Rev Cardiol 2011 Mar;8(3):131-139.
(30) Loftus IM, Naylor AR, Goodall S, Crowther M, Jones L, Bell PR, et al. Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. Stroke 2000 Jan;31(1):40-47.
(31) Choudhary S, Higgins CL, Chen IY, Reardon M, Lawrie G, Vick GW,3rd, et al. Quantitation and localization of matrix metalloproteinases and their inhibitors in human carotid endarterectomy tissues. Arterioscler Thromb Vasc Biol 2006 Oct;26(10):2351-2358.
(32) Prasad K. Low levels of serum soluble receptors for advanced glycation end products, biomarkers for disease state: myth or reality. Int J Angiol 2014 Mar;23(1):11-16.
(33) Park HJ, Baek JY, Shin WS, Kim DB, Jang SW, Shin DI, et al. Soluble receptor of advanced glycated endproducts is associated with plaque vulnerability in patients with acute myocardial infarction. Circ J 2011;75(7):1685-1690.
(34) Ishibashi T, Kawaguchi M, Sugimoto K, Uekita H, Sakamoto N, Yokoyama K, et al. Advanced glycation end product-mediated matrix metallo-proteinase-9 and apoptosis via renin-angiotensin system in type 2 diabetes. J Atheroscler Thromb 2010 Jun 30;17(6):578-589.

(35) Cipollone F, Iezzi A, Fazia M, Zucchelli M, Pini B, Cuccurullo C, et al. The receptor RAGE as a progression factor amplifying arachidonate-dependent inflammatory and proteolytic response in human atherosclerotic plaques: role of glycemic control. Circulation 2003 Sep 2;108(9):1070-1077.
(36) Hettema ME, Bootsma H, Graaff R, de Vries R, Kallenberg CG, Smit AJ. Skin autofluorescence, as marker of accumulation of advanced glycation endproducts and of cumulative metabolic stress, is not increased in patients with systemic sclerosis. Int J Rheumatol 2011;2011:417813.
(37) de Groot L, Hinkema H, Westra J, Smit AJ, Kallenberg CG, Bijl M, et al. Advanced glycation endproducts are increased in rheumatoid arthritis patients with controlled disease. Arthritis Res Ther 2011 Dec 14;13(6):R205.
(38) Ninomiya T, Zoungas S, Neal B, Woodward M, Patel A, Perkovic V, et al. Efficacy and safety of routine blood pressure lowering in older patients with diabetes: results from the ADVANCE trial. J Hypertens 2010 Jun;28(6):1141-1149.
(39) Zoungas S, Patel A, Chalmers J, de Galan BE, Li Q, Billot L, et al. Severe hypoglycemia and risks of vascular events and death. N Engl J Med 2010 Oct 7;363(15):1410-1418.
(40) Bao W, Min D, Twigg SM, Shackel NA, Warner FJ, Yue DK, et al. Monocyte CD147 is induced by advanced glycation end products and high glucose concentration: possible role in diabetic complications. Am J Physiol Cell Physiol 2010 Nov;299(5):C1212-9.
(41) Berrou J, Tostivint I, Verrecchia F, Berthier C, Boulanger E, Mauviel A, et al. Advanced glycation end products regulate extracellular matrix protein and protease expression by human glomerular mesangial cells. Int J Mol Med 2009 Apr;23(4):513-520.
(42) Sun Z, Liu J, Zeng X, Huangfu J, Jiang Y, Wang M, et al. Protective actions of microalgae against endogenous and exogenous advanced glycation endproducts (AGEs) in human retinal pigment epithelial cells. Food Funct 2011 May;2(5):251-258.
(43) Navaratna D, McGuire PG, Menicucci G, Das A. Proteolytic degradation of VE-cadherin alters the blood-retinal barrier in diabetes. Diabetes 2007 Sep;56(9):2380-2387.

Part 2

IMAGING OF ATHEROSCLEROSIS

CHAPTER 5

Macrophage folate receptor β (FR-β) expression in auto-immune inflammatory rheumatic diseases: a forthcoming marker for cardiovascular risk?

Nynke A. Jager, Nato Teteloshvili, Clark J. Zeebregts, Johanna Westra, Marc Bijl

Autoimmun Rev. 2012 Jul;11 (9):621-6.

Abstract

In patients with systemic auto-immune inflammatory rheumatic diseases (AIIRD) like rheumatoid arthritis the prevalence of cardiovascular disease (CVD) is increased. In the pathogenesis of AIIRD and atherosclerosis many similarities can be found in the process underlying CVD. Accumulation of inflammatory cells, in particular macrophages at the site of inflammation producing inflammatory mediators serve as a prominent feature in both systemic inflammation and atherosclerosis.

Two different subtypes of macrophages have been described in recent literature namely classically activated macrophages (M1) and alternatively activated macrophages (M2). Alternatively activated macrophages are characterized by low CD14 and high CD163 expression. Macrophages expressing CD14 (M1) have been identified within atherosclerotic plaques, whereas CD14 low macrophages are abundant in vessels without atherosclerosis. Depending on the environment and responses to different stimuli, macrophages in plaques can express diverse pro and anti- atherogenic functions. The balance of these different activation profiles influences atheroma evolution and outcome.

Nowadays, influx of macrophages is recognized as a very important feature of the pathogenesis of plaque formation. Activated macrophages accumulate at the sites of inflammation and can therefore be exploited to better visualize inflammatory responses in atherosclerosis. Furthermore, activated (but not resting) macrophages possess a functionally active receptor for folate (FR-β), but it is not completely clear which subtype of this activated macrophages expresses this receptor and whether the expression of FR-β is restricted to only one of the macrophage subsets. Although future research needs to be done to investigate FR-β expression and function within inflamed tissues, the expression of functional FR-β on tissue macrophages likely occurs during activation. Therefore, expression of FR-β on activated macrophages holds a promising potential for early diagnosis and better analysis of optimal treatment regiments of vascular diseases in association with systemic diseases.

Keywords: auto-immune inflammatory rheumatic diseases; atherosclerosis; macrophage plasticity; vulnerable plaques; FR-β

Highlights:

- Macrophages play an important role in the pathogenesis of both AIIRD and atherosclerosis and might link these diseases
- Macrophages can be distinguished in M1 and M2 subtypes
- The balance between M1 and M2 macrophages will influence plaque vulnerability
- FR-β, a marker for M2 macrophages, could be a tool for early detection of atherosclerosis

1. Atherosclerosis in patients with systemic inflammation

1.1 General introduction

Until a view decades ago, atherosclerosis was thought to be a collection of cholesterol and accumulated smooth muscle cells within the arterial vessel wall. In the 1960s, macrophages were identified in atherosclerotic plaques (1) but investigations into the pathophysiology of this condition focused on diet and metabolism, as there was overwhelming evidence that cholesterol leads to the development of atherosclerosis (2). Although an additional link between adipose tissue and rheumatoid arthritis was found (3), inflammation, another player in the field may even have more influence on accelerating atherosclerosis. The importance of inflammation in arterial disease was first pointed out by Virchow (4). New insights into the importance of inflammatory cells in the pathogenesis of atherosclerosis emerged, based on the observation of similarities between atherosclerosis and systemic auto-immune inflammatory rheumatic diseases (AIIRD). Various autoimmune rheumatic diseases such as rheumatoid arthritis (RA) and SLE (systemic lupus erythematosus) accelerate atherosclerosis. (5) Early in the atherosclerotic process as well as at sites of inflammation endothelial cells change from an anti-inflammatory phenotype that resist adhesion and promote fibrinolysis, into a pro-inflammatory phenotype. The latter, activated endothelial cells are characterized by increased expression of endothelin and leukocyte adhesion molecules (ICAM-1, VCAM-1, E-selectin) (6). This facilitates binding and subsequent infiltration and activation of inflammatory cells (in particular monocytes, T-cells and mast cells), and production of pro-inflammatory cytokines such as tumor necrosis factor (TNF-alpha) and interleukin (IL)-6 (7,8). Chemotactic stimuli, provided by pro-inflammatory proteins, such as chemo attractant protein-1 (MCP-1), further promote the recruitment of monocytes by diapedesis through interaction with different receptors, such as monocyte chemokine receptor CCR2 (6). Within the intima, monocytes change into macrophages with help of the monocyte maturation mediator macrophage colony-stimulating factor (M-CSF). M-CSF can induce scavenger receptor expression and as such promote a differential shift from monocytes into macrophages (9). Macrophages internalize oxLDL by the macrophage scavenger receptors which lead to the formation of foam cells (10) (Figure 1).

1.2 Pathogenesis of atherosclerosis and inflammation; the importance of the macrophage

Increased numbers of macrophages producing inflammatory mediators in the synovial fluid are a prominent feature in patients with AIIRD. In atherosclerotic plaques, also a higher number of macrophages is present and this feature supposedly links both diseases (11). Interestingly, in patients with RA, the primary site of inflammation is synovial tissue, from which cytokines can be released into the systemic circulation. These circulating cytokines alter function of distant tissues, in order to generate a spectrum of pro-atherogenic changes

including endothelial dysfunction and damage rendering patients at risk for overt CVD (12-14). For example, TNF-α, secreted by activated macrophages is increased in plasma of SLE patients with CVD, compared to age-matched patients without manifest CVD (15). The above mentioned facts indicate the importance of inflammation, and in particular macrophage activation in the development of atherosclerosis in patients with AIIRD.

Macrophages interact with cells in the vessel wall including T-cells and vascular smooth muscle cells (VSMC), directly or via release of cytokines. This interaction results in an enhanced inflammatory response due to subsequent cell activation. Activation induced cell death, through apoptosis, may occur. Apoptosis of macrophages appears to result from cell–cell interactions and the local cytokine environment within the arterial wall, involving the actions of pro- and anti-apoptotic proteins that include death receptors, proto-oncogenes, and tumor suppressor genes (12). Apoptotic macrophages are rapidly cleared by phagocytosis. However, when this process is insufficient, apoptotic cells will turn into secondary necrotic cells with accumulation of insoluble lipids and other cellular contents, a characteristic of advanced lesions (16). Macrophages will also release metalloproteinases (MMPs) which degrade the extracellular matrix (ECM) proteins. MMPs lead to plaque instability and rupture (17-19). Apart from being the source of MMPs, macrophages have been strongly implicated in atherosclerotic plaque rupture through several other mechanisms. Production of death ligands, reactive oxygen species including nitric oxide and proteases can cause apoptosis of SMC (20), which in turn leads to the fibrous cap thinning (21). Also, death of foam cell macrophages (FCM) provokes lipid core expansion, especially in advanced plaques. Finally, macrophages stimulate invasion of vasa vasorum into a base of the plaque, which will further promote intraplaque hemorrhage due to growth of the core (22). Plaque rupture leads to the activation of the coagulation cascade, fibrin deposition, platelet activation and recruitment to form a thrombus (16,23).

1.3 Heterogeneous subpopulations of macrophages within atherosclerotic plaques

Macrophages have been identified as a heterogeneous population of cells with a variety of physiological and pathophysiological functions (24,25). Stein and colleagues were the first to describe ''classically'' (M1) activated macrophages as having a different phenotype from what are now called ''alternatively'' (M2) activated macrophages (26) . M1 macrophage polarization is induced mainly by bacterial moieties such as LPS and the Th1 cytokine interferon–gamma (IFN-γ). M1 macrophages contribute to the inflammatory response and tissue damage by producing copious amounts of reactive oxygen species, nitrogen intermediates and pro-inflammatory cytokines such as TNF-alpha and IL-6 (24,27). In contrast, ''alternatively'' activated M2 macrophages originate from monocytes upon stimulation with Th2 cytokines, such as IL-4 (26), as depicted in Figure 1.

Figure 1.

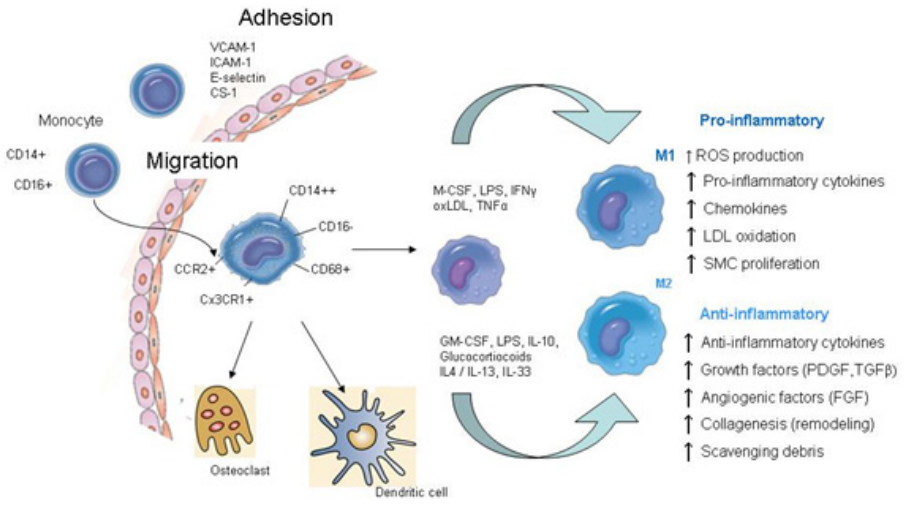


Figure 1. Subsets of activated macrophages in human atherosclerotic plaques.
Monocytes migrate into the developing atheroma lesion guided by chemokines and adhesion molecules. Infiltrated monocytes become macrophages or differentiate into dendritic cells or osteoclasts. Macrophages in response to the different stimuli further differentiate into M1 (pro-inflammatory type) macrophage which promotes the lesion development or M2 (anti-inflammatory type) that are immune regulatory and upon the stimuli modify the development of atherosclerotic plaques.

In contrast to M1 macrophages, M2 macrophages display a Th2–like phenotype, promoting ECM construction, cell proliferation and angiogenesis (28,29). The precise properties of M2 macrophages vary, depending on their activating conditions, and M2 macrophages have been divided into M2a, M2b and M2c subsets (25) (Table 1).

Table 1. Comparison of distinct macrophage subpopulations

	M1	**M2a**	**M2b**	**M2c**
Activating signals Biological markers	IFN-γ, LPS, TLR, TNF-α, IL-1, CpG-DNA CD80/86 ↑, IL-2, IL-12^{hi}, IL-10^{lo}, IL-23^{hi}, mannose receptor↓, MHC II↑	IL-4, IL-13, CCL2, CXCL4, PPAR-γ (?) Mannose receptor↑, CD23↑, CD14↓, CD163, CCR4, CCR3, IL-4R-α, FR-β (?)	LPS, CD40 ligand, IL-1β, CD206, CD163, PPAR-γ (?)	IL-10, TGF-β, ucocorticosteroids, IL-1R, TLR-4, FcγRI, FcγRIIa and FcγRIII, PPAR-γ (?)
Secretory products	NO, nitrogen intermediates, TNF-α, IL-6, tissue degrading proteases, IL-1, iNOS, arginase II	TGF-β, dectin-1, mannose-binding lectin, IL-1Rα, NO, IL10, IL-12, arginase I, polyamines, iron	IL-10, IL12, TNF-α, IL-6, IL-1β	IL-10, IL-12, TNF-α
Chemokine secretion	MCP-1, MIP-1α, IP-10, CXCL9, CXCL10	AMAC-1, CCL17, CCL22, CCL24	CCl18^{hi}, CCl16^{hi}, CCL3^{lo}	CCL1^{hi}, PGE2^{hi}
Function	Enhanced phagocytic capacity Promotion of Th1 responses Tumor destruction Tissue damage	Promotion of Th2 response Clearance of parasites Immuneregulation Pro-tumor activity ovascularization↑) Tissue remodeling Profibrotic (ECM deposition↑) Folate uptake, metabolism	Immuneregulation Tumor promotion	Debris scavenging activity Pro-healing Immuneregulation Tumor progression

Although the phenotype and the functions of anti- and pro-inflammatory macrophages are different, both subsets are important components for the innate and adaptive immune response.

Interestingly, nowadays there is a growing body of evidence suggesting presence of a heterogeneous population of macrophages in human atherosclerotic lesions (30-33). Initially, M1 and M2 could be hardly discriminated in plaque tissue due to lack of appropriate markers to distinguish these specific macrophage subsets in tissue specimens. Later on, CD163, the haptoglobin / haemoglobin scavenger receptor was identified as a marker for alternatively activated macrophages (32,34). These M2 macrophages were diffusely present within intimal atherosclerotic lesions (28). CD163 positive macrophages also have been identified in haemorrhaged atherosclerotic plaques. They expressed low

levels of human leukocyte antigen- DR (HLA- DR) and were unlikely classical lipid core macrophages, because CD163 is a marker for alternatively activated macrophages (34). Hence, these macrophage types were thought to suppress the impact of haemorrhage on atherosclerotic progression. M2 macrophages have been also characterized by low CD14 (monocyte differentiation antigen) expression. Macrophages with low levels of CD14 express 5 times more PPAR gamma than macrophages expressing high levels of CD14, and PPAR gamma is associated with an M2 – anti-inflammatory phenotype (33). Regarding the low CD14 expression and M2 macrophage activation, Waldo and colleagues have shown that CD14 and CD68 double positive macrophages could be identified within atherosclerotic plaques, whereas, $CD14^{low}$ macrophages are abundant in normal intimal regions of coronary arteries (31). A recent study by Khallou-Laschet et al. evaluated the phenotype of macrophages associated with progression of atherosclerosis in the apoplipoprotein E (ApoE) knockout mouse model. Their study demonstrated that bone marrow-derived macrophages submitted to M1 and M2 polarization specifically expressed arginase II and I, respectively. They also found lesion progression in atherosclerotic disease was correlated with the dominance of M1 over the M2 macrophage subtype (35). M-CSF differentiated macrophages (M2 subtype) express all three activating Fc gamma receptors (FcγR); FcγRIII, FcγRIIa and FcγRI (CD16, CD32 and CD64 respectively) (36-38). Though opinions are divided about this, concerning Lennartz et al. found M1 macrophages express elevated levels of FcγRI, FcγRIIa and FcγRIII, while M2 macrophages express a lower amount of these receptors (39). Given the complexity of atherosclerosis, it is difficult to generalize the findings obtained from murine model in human situation. However, these observations provide evidence that plaque pathogenesis and evolution are influenced by macrophage activation and polarization. Depending on environmental factors and responses to different stimuli, macrophages in plaques can express diverse pro- and anti-atherogenic functions. As a result, the balance of these different activation profiles will influence atheroma evolution and outcome. Recently, folate receptor-β expression (FR-β) has been found on M-CSF polarized M2 macrophages. As such FR-β might serve as a new marker for M2 macrophages (40).

2. Folate receptor-β

2.1 Structure and function of FR-β

Folate (vitamin B9 or folic acid) is critical for the synthesis of nucleotide bases via carrying the methyl groups within cells. Folates are hydrophilic anionic molecules that do not cross biological membranes by diffusion alone, so sophisticated membrane transport systems have evolved for facilitating folate accumulation by mammalian cells and tissues. In mammalian cells, folate is transported through two major mechanisms. One involves a transmembrane transporter protein, which is the reduced folate carrier (a member of the SLC19 family of facilitative carriers) which contributes to anion exchange with high affinity for low folate. The other implies proton coupled folate transporter which belong to the membrane–spanning proteins and mediate bidirectional transfer of reduced folate across the plasma membrane (41,42).

Folate can be internalized by the folate receptor (FR) that is expressed on a limited number of cells and can mediate unidirectional transport of folate into the cells. Three major forms of the folate receptor exist: FR-α, FR-β and FR-γ (43). Among these, the β isoform belongs to the glycosylphosphatidylinositol (GPI) anchored proteins that binds to folate with high affinity (KD<1 nM). FR-β quantitatively recycles, within minutes, between the cell surface and intracellular compartments (44). FR-β is of great importance when folate supply is low or rapid cell growth requires elevated uptake of folate. FR-β is mostly expressed in hematopoetic cells of myelomonocytic lineages (45-47).

2.2 FR-β as a selective cellular marker for activated macrophages

Expression of FR on nonmalignant hematopoetic cells (primarily the beta isoform) appears to be limited to activated macrophages and their precursors (48,49). Previous studies in animal models of RA have shown that activated macrophages express FR-β (40), in particular M2 alternatively activated macrophages (50). The quantitation of activated macrophages in systemic inflammation, in particular RA joint tissues might consequently be of diagnostic value, because activated macrophages content correlates well with articular destruction and poor prognosis in human (51). Since FR expression coincides with macrophage activation, it seems reasonable to image arthritic joints using folate-derived imaging agents. Turk et al. demonstrated that ^{99m}Tc-folate can indeed selectively target activated macrophages *in vivo*, in an adjuvant-induced arthritis mouse model, and that folate-linked imaging agents can facilitate the non-invasive analysis of inflammatory activity (51).

Nowadays, presence of (coronary) atherosclerosis (in RA patients) can be determined using recently developed imaging techniques such as echocardiographic measurement of coronary flow reserve and tissue Doppler imaging, which can detect subtle cardiovascular abnormalities. (52). Because atherosclerosis is also characterized by a localized accumulation of activated macrophages, it is important to determine whether folate conjugates might

prove useful in imaging atherosclerosis as well. Using apolipoprotein E knockout (apoE-/-) mice raised on high-fat diet, Ayala-Lopez et al. documented selective uptake of ^{99m}Tc-EC20 by macrophages present within atherosclerosis lesions by whole-animal radio imaging (53). Consequently, folate conjugates proved to be useful also in imaging atherosclerosis progression *in vivo*. Although progress has been made in employing FR- targeted imaging of macrophages within atherosclerotic plaques, the findings obtained from mouse studies are difficult to extrapolate to humans. Very recently, Low and colleagues developed an antibody with high specificity to human FR-β and demonstrated that this antibody is able to target FR- β positive cells including macrophages from RA patients (54). The given findings suggest that high affinity FR- β specific antibody can be applied as a research tool for effective targeting of activated macrophages during inflammatory disease progression, which can be used also for early detection of atherosclerosis.

Recent evidence suggests that FR- β expression is not limited only to activated macrophages. It is also present in membrane lysates and on the cell surface of M2 macrophages generated in the presence of M-CSF. The existence of FR-β in M-CSF primed *in vitro* macrophages is in agreement with its up-regulation in human uterine macrophages that exhibit an immunosuppressive phenotype and whose gene expression profile closely corresponds to that of M2- polarized phenotype (55). The presence of functional FR- β on M-CSF primed macrophages is difficult to reconcile with its expression and function in synovial macrophages from RA patients, which are embedded in an inflammatory environment. So far, few studies have specifically examined the role of M2 in pathogenesis of RA and further studies are needed to correlate FR- β expression and function in macrophage subsets within inflamed tissues. However, it is tempting to speculate that synovial tissue of RA patients might exhibit a mixture of M1 and M2 phenotype and an explanation that would be compatible with the high levels of M-CSF generated macrophages found in RA patients synovia (56).

Like in RA, heterogeneous macrophage populations have been also reported within atherosclerotic lesions and their phenotype has been correlated with plaque stability (29). Because FR expression has been linked to macrophage activation and M2 differentiation, it will be informative to explore whether the abundance of FR-positive macrophages at sites of atherosclerotic lesions is associated with plaque vulnerability. If such association emerges, it would support further exploration of the use of FR-targeted agents for early detection of unstable plaques in chronic inflammatory diseases, hence identifying individuals at risk for overt CVD.

3. Challenges and future prospects

Patients with atherosclerosis are at high risk to develop cardiovascular disease already at disease onset (57). This increased risk cannot be fully explained by traditional cardiovascular risk factors alone (58). The current paradigm places inflammation at the very heart of the

pathogenesis of atherosclerosis. The presence of chronic inflammation in autoimmune diseases such as RA appears to be an important source of cellular and molecular processes that drives the development of atherosclerotic lesions. Macrophages which play a crucial role in the development of chronic inflammatory conditions are present in all stages of atherogenesis, from the nascent fatty streak lesion to the culmination in cardiovascular events. Nowadays medicine face challenges in establishing the algorithms for cardiovascular disease risk assessment based on high chronic inflammatory burden. Challenges appear in early diagnosis of atherosclerosis in patients with autoimmune diseases.

Since plaque rupture is a major cause of cardiovascular events, attention has been directed on evaluation of biological processes that determine plaque vulnerability. A number of non-invasive imaging techniques have been developed to evaluate vascular wall in autoimmune diseases in an attempt to identify so-called vulnerable atherosclerotic plaques which are prone to rupture (59). In recent years, the potential of molecular imaging to characterize the process involved in the initiation and progression of atherosclerotic lesions has been explored. In particular, radionuclide-based molecular imaging, intravascular ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) have been evaluated in preclinical and clinical studies. Despite their high sensitivity and accuracy in detection of plaque vulnerability, substantial technical and methodological constraints remain unsolved, such as time- consuming preparation, slow plasma clearance, spatial resolution and high costs (59, 60). As such, much remains to be improved before their wide application in clinics.

Currently different macrophage subpopulations have been identified within chronic inflammatory diseases as well in atherosclerosis. It has been proposed that macrophage polarization is not irreversible and macrophages can switch between M1 and M2 phenotype, which is referred as macrophage plasticity [23, 24]. That would suggest that cells that initially promote an inflammatory response (M1) could have later anti-inflammatory properties (35). So far, classically activated macrophages seem the most obvious culprits in atherosclerotic plaque disruption and hence acute coronary events because they have an enhanced ability to kill neighboring cells and destroy extracellular matrix (24). Subsequently, alternatively activated M2 macrophages could suppress the impact of hemorrhage on atherosclerosis progression. However, the effects of M2 macrophages in vulnerable lesions are by definition too little and too late (24,32). Due to the lack of the markers that identify macrophage populations involved in the pathogenesis of autoimmune disorders and lack of knowledge about their precise functions, little is known about to what extend subsets of macrophages contribute to the subsequent atherosclerotic plaque progression. The primary issues concern the plasticity of the macrophages *in vivo*, correlations between *in vitro* polarization and their *in vivo* behavior, what is serving as robust marker for macrophage polarization in disease and how polarization can be manipulated to alter the outcome of chronic inflammatory conditions (16,24). Elucidating the exact role of macrophage subpopulations within lesions and the

molecular clues that drive differentiation and activation, would undoubtedly aid in the development of novel strategies for early diagnosis, stabilization or even regression of vulnerable atherosclerotic plaques.

With recent discovery of FR-beta expression on activated macrophages, folate targeting and imaging of chronic inflammatory disorders and atherosclerosis can now be envisioned (48,49). Expression of FR-beta on distinct sets of the macrophages could present an important morphological marker for the detection of the patients who are at high-risk for developing atherosclerotic plaques. Assessment of FR-beta positive macrophages among the individuals with chronic inflammatory diseases may aid in identification of the patients with vulnerable plaques non- invasively. Hence, FR-beta targeted imaging may harbor tremendous potential in clinical applications in a large population of subjects at risk of cardiovascular events.

Concluding remarks (take home message)

The fact that macrophage subsets can be employed as biomarkers for chronic inflammatory diseases and that they might represent treatment targets will undoubtedly continue to provide fertile ground for further investigation. Some exogenous cell trackers / reagents such as antibody against FR-β would selectively image macrophage subset activity associated with atherosclerotic plaque progression that may be clinically translatable. Therefore it may be useful to employ FR- β as *in vivo* imaging tool to non-invasively and rapidly assess prognosis of atherosclerosis in patients with chronic inflammatory diseases and implicate it as a forthcoming marker for cardiovascular risk detection. Moreover, this approach might determine whether a direct relation between progression of atherosclerosis and disease activity exists, and whether this effect can be influenced by therapeutic intervention.

References

(1) ANITSCHKOW N. On the morphodynamics of coronary sclerosis of the heart. Acta Pathol Microbiol Scand 1960;49:426-432.
(2) Hansson GK. Atherosclerosis--an immune disease: The Anitschkov Lecture 2007. Atherosclerosis 2009 Jan;202(1):2-10.
(3) Gremese E, Ferraccioli G. The metabolic syndrome: the crossroads between rheumatoid arthritis and cardiovascular risk. Autoimmun Rev 2011 Aug;10(10):582-589.
(4) Mayerl C, Lukasser M, Sedivy R, Niederegger H, Seiler R, Wick G. Atherosclerosis research from past to present--on the track of two pathologists with opposing views, Carl von Rokitansky and Rudolf Virchow. Virchows Arch 2006 Jul;449(1):96-103.
(5) Nussinovitch U, Shoenfeld Y. Atherosclerosis and macrovascular involvement in systemic sclerosis: myth or reality. Autoimmun Rev 2011 Mar;10(5):259-266.
(6) Cybulsky MI, Gimbrone MA,Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 1991 Feb 15;251(4995):788-791.
(7) Pasceri V, Yeh ET. A tale of two diseases: atherosclerosis and rheumatoid arthritis. Circulation 1999 Nov 23;100(21):2124-2126.
(8) van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 1994 Jan;89(1):36-44.
(9) Clinton SK, Underwood R, Hayes L, Sherman ML, Kufe DW, Libby P. Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. Am J Pathol 1992 Feb;140(2):301-316.
(10) Pennings M, Meurs I, Ye D, Out R, Hoekstra M, Van Berkel TJ, et al. Regulation of cholesterol homeostasis in macrophages and consequences for atherosclerotic lesion development. FEBS Lett 2006 Oct 9;580(23):5588-5596.
(11) Smeets TJ, Kraan MC, Galjaard S, Youssef PP, Smith MD, Tak PP. Analysis of the cell infiltrate and expression of matrix metalloproteinases and granzyme B in paired synovial biopsy specimens from the cartilage-pannus junction in patients with RA. Ann Rheum Dis 2001 Jun;60(6):561-565.
(12) Sitia S, Tomasoni L, Atzeni F, Ambrosio G, Cordiano C, Catapano A, et al. From endothelial dysfunction to atherosclerosis. Autoimmun Rev 2010 Oct;9(12):830-834.
(13) Zardi EM, Afeltra A. Endothelial dysfunction and vascular stiffness in systemic lupus erythematosus: Are they early markers of subclinical atherosclerosis? Autoimmun Rev 2010 Aug;9(10):684-686.
(14) Sattar N, McCarey DW, Capell H, McInnes IB. Explaining how "high-grade" systemic inflammation accelerates vascular risk in rheumatoid arthritis. Circulation 2003 Dec 16;108(24):2957-2963.
(15) Svenungsson E, Jensen-Urstad K, Heimburger M, Silveira A, Hamsten A, de Faire U, et al. Risk factors for cardiovascular disease in systemic lupus erythematosus. Circulation 2001 Oct 16;104(16):1887-1893.
(16) Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell 2001 Feb 23;104(4):503-516.
(17) Moulton KS, Heller E, Konerding MA, Flynn E, Palinski W, Folkman J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. Circulation 1999 Apr 6;99(13):1726-1732.
(18) Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest 1994 Dec;94(6):2493-2503.
(19) Newby AC. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. Physiol Rev 2005 Jan;85(1):1-31.
(20) Boyle JJ, Weissberg PL, Bennett MR. Human macrophage-induced vascular smooth muscle cell apoptosis requires NO enhancement of Fas/Fas-L interactions. Arterioscler Thromb Vasc Biol 2002

Oct 1;22(10):1624-1630.
(21) Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. J Am Coll Cardiol 2006 Apr 18;47(8 Suppl):C13-8.
(22) Clarke MC, Figg N, Maguire JJ, Davenport AP, Goddard M, Littlewood TD, et al. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. Nat Med 2006 Sep;12(9):1075-1080.
(23) Husmann M, Barton M. Therapeutical potential of direct thrombin inhibitors for atherosclerotic vascular disease. Expert Opin Investig Drugs 2007 May;16(5):563-567.
(24) Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005 Dec;5(12):953-964.
(25) Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci 2008 Jan 1;13:453-461.
(26) Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med 1992 Jul 1;176(1):287-292.
(27) Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. J Leukoc Biol 2004 Sep;76(3):509-513.
(28) Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 2009;27:451-483.
(29) Gordon S. Alternative activation of macrophages. Nat Rev Immunol 2003 Jan;3(1):23-35.
(30) Poston RN, Hussain IF. The immunohistochemical heterogeneity of atheroma macrophages: comparison with lymphoid tissues suggests that recently blood-derived macrophages can be distinguished from longer-resident cells. J Histochem Cytochem 1993 Oct;41(10):1503-1512.
(31) Waldo SW, Li Y, Buono C, Zhao B, Billings EM, Chang J, et al. Heterogeneity of human macrophages in culture and in atherosclerotic plaques. Am J Pathol 2008 Apr;172(4):1112-1126.
(32) Boyle JJ, Harrington HA, Piper E, Elderfield K, Stark J, Landis RC, et al. Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. Am J Pathol 2009 Mar;174(3):1097-1108.
(33) Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S, et al. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab 2007 Aug;6(2):137-143.
(34) Komohara Y, Hirahara J, Horikawa T, Kawamura K, Kiyota E, Sakashita N, et al. AM-3K, an anti-macrophage antibody, recognizes CD163, a molecule associated with an anti-inflammatory macrophage phenotype. J Histochem Cytochem 2006 Jul;54(7):763-771.
(35) Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, Clement M, et al. Macrophage plasticity in experimental atherosclerosis. PLoS One 2010 Jan 25;5(1):e8852.
(36) Leidi M, Gotti E, Bologna L, Miranda E, Rimoldi M, Sica A, et al. M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells in vitro. J Immunol 2009 Apr 1;182(7):4415-4422.
(37) Mosser DM. The many faces of macrophage activation. J Leukoc Biol 2003 Feb;73(2):209-212.
(38) Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 2010 Oct;11(10):889-896.
(39) Lennartz MR, Aggarwal A, Michaud TM, Feustel PJ, Jones DM, Brosnan MJ, et al. Ligation of macrophage Fcgamma receptors recapitulates the gene expression pattern of vulnerable human carotid plaques. PLoS One 2011;6(7):e21803.
(40) Paulos CM, Varghese B, Widmer WR, Breur GJ, Vlashi E, Low PS. Folate-targeted immunotherapy effectively treats established adjuvant and collagen-induced arthritis. Arthritis Res Ther 2006;8(3):R77.
(41) Matherly LH, Hou Z, Deng Y. Human reduced folate carrier: translation of basic biology to cancer etiology and therapy. Cancer Metastasis Rev 2007 Mar;26(1):111-128.

(42) Matherly LH, Goldman DI. Membrane transport of folates. Vitam Horm 2003;66:403-456.
(43) Elnakat H, Ratnam M. Distribution, functionality and gene regulation of folate receptor isoforms: implications in targeted therapy. Adv Drug Deliv Rev 2004 Apr 29;56(8):1067-1084.
(44) Rothberg KG, Ying YS, Kolhouse JF, Kamen BA, Anderson RG. The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. J Cell Biol 1990 Mar;110(3):637-649.
(45) Reddy JA, Haneline LS, Srour EF, Antony AC, Clapp DW, Low PS. Expression and functional characterization of the beta-isoform of the folate receptor on CD34(+) cells. Blood 1999 Jun 1;93(11):3940-3948.
(46) Ross JF, Wang H, Behm FG, Mathew P, Wu M, Booth R, et al. Folate receptor type beta is a neutrophilic lineage marker and is differentially expressed in myeloid leukemia. Cancer 1999 Jan 15;85(2):348-357.
(47) Ratnam M, Marquardt H, Duhring JL, Freisheim JH. Homologous membrane folate binding proteins in human placenta: cloning and sequence of a cDNA. Biochemistry 1989 Oct 3;28(20):8249-8254.
(48) Xia W, Hilgenbrink AR, Matteson EL, Lockwood MB, Cheng JX, Low PS. A functional folate receptor is induced during macrophage activation and can be used to target drugs to activated macrophages. Blood 2009 Jan 8;113(2):438-446.
(49) van der Heijden JW, Oerlemans R, Dijkmans BA, Qi H, van der Laken CJ, Lems WF, et al. Folate receptor beta as a potential delivery route for novel folate antagonists to macrophages in the synovial tissue of rheumatoid arthritis patients. Arthritis Rheum 2009 Jan;60(1):12-21.
(50) Puig-Kroger A, Sierra-Filardi E, Dominguez-Soto A, Samaniego R, Corcuera MT, Gomez-Aguado F, et al. Folate receptor beta is expressed by tumor-associated macrophages and constitutes a marker for M2 anti-inflammatory/regulatory macrophages. Cancer Res 2009 Dec 15;69(24):9395-9403.
(51) Matteson EL, Lowe VJ, Prendergast FG, Crowson CS, Moder KG, Morgenstern DE, et al. Assessment of disease activity in rheumatoid arthritis using a novel folate targeted radiopharmaceutical Folatescan. Clin Exp Rheumatol 2009 Mar-Apr;27(2):253-259.
(52) Atzeni F, Turiel M, Hollan I, Meroni P, Sitia S, Tomasoni L, et al. Usefulness of cardiovascular biomarkers and cardiac imaging in systemic rheumatic diseases. Autoimmun Rev 2010 Oct;9(12):845-848.
(53) Ayala-Lopez W, Xia W, Varghese B, Low PS. Imaging of atherosclerosis in apoliprotein e knockout mice: targeting of a folate-conjugated radiopharmaceutical to activated macrophages. J Nucl Med 2010 May;51(5):768-774.
(54) Feng Y, Shen J, Streaker ED, Lockwood M, Zhu Z, Low PS, et al. A folate receptor beta-specific human monoclonal antibody recognizes activated macrophage of rheumatoid patients and mediates antibody-dependent cell-mediated cytotoxicity. Arthritis Res Ther 2011 Apr 8;13(2):R59.
(55) Gustafsson C, Mjosberg J, Matussek A, Geffers R, Matthiesen L, Berg G, et al. Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype. PLoS One 2008 Apr 30;3(4):e2078.
(56) Pollard JW. Trophic macrophages in development and disease. Nat Rev Immunol 2009 Apr;9(4):259-270.
(57) Bartoloni E, Alunno A, Bistoni O, Gerli R. How early is the atherosclerotic risk in rheumatoid arthritis? Autoimmun Rev 2010 Aug;9(10):701-707.
(58) Sarzi-Puttini P, Atzeni F, Gerli R, Bartoloni E, Doria A, Barskova T, et al. Cardiac involvement in systemic rheumatic diseases: An update. Autoimmun Rev 2010 Oct;9(12):849-852.
(59) Perrone-Filardi P, Dellegrottaglie S, Rudd JH, Costanzo P, Marciano C, Vassallo E, et al. Molecular imaging of atherosclerosis in translational medicine. Eur J Nucl Med Mol Imaging 2011 May;38(5):969-975.

(60) ten Kate GL, Sijbrands EJ, Staub D, Coll B, ten Cate FJ, Feinstein SB, et al. Noninvasive imaging of the vulnerable atherosclerotic plaque. Curr Probl Cardiol 2010 Nov;35(11):556-591.

CHAPTER 6

Targeted folate receptor β fluorescence imaging as a measure of inflammation to estimate vulnerability within the human atherosclerotic carotid plaque

Nynke A. Jager, Johanna Westra, Gooitzen M. van Dam, Nato Teteloshvili, René A. Tio, Jan-Cees Breek, Riemer H.J.A. Slart, Hendrikus Boersma, Phillip S. Low, Marc Bijl, Clark J. Zeebregts

J Nucl Med. 2012 Aug;53 (8):1222-9.

Abstract

OBJECTIVES The probability of atherosclerotic plaque rupture and its thrombotic sequelae are thought to be increased at sites of macrophage accumulation. Folate receptor β (FR-β) is present on activated macrophages but not on quiescent macrophages or other immune cells. By conjugating the ligand folate with a fluorescent contrast agent, fluoresceine-isothiocynate (FITC), we aimed to explore the potential role of folate receptor β fluorescence imaging in the distinction of vulnerable sites from more stable regions.

METHODS Carotid specimen were taken from a total of 20 patients and incubated with folate-FITC for 30 minutes. *Ex vivo* fluorescence imaging with an IVIS Spectrum® was performed to determine the exact location of folate-FITC uptake. Sections displaying regions of high uptake (determined as hot spots) were compared with sections showing low uptake (cold spots) through immunohistochemistry and real time quantitative reverse transcription PCR for FR-β.

RESULTS Hot spots showed significantly higher folate-FITC uptake compared to cold spots ($p<0.001$). Hot spots tended to contain more macrophages and areas of hypoxia compared to cold spots. A positive correlation between mRNA levels of CD68 (marker for macrophages), folate receptor β ($r=0.53$, $p=0.045$) and HIF-1α expression (marker for intraplaque hypoxia; $r=0.55$, $p=0.034$) was found.

CONCLUSION In areas of high folate-FITC uptake within human atherosclerotic plaques, an increased number of activated macrophages and higher areas of hypoxia were present compared to areas with low folate-FITC uptake. These characteristics of vulnerability imply that molecular imaging of folate receptor-β through folate conjugates might be a good indicator for plaque vulnerability in future non-invasive imaging studies.

Keywords: Carotid artery; atherosclerotic plaque; vulnerability; folate receptor-β; fluorescence imaging.

Introduction

Atherosclerosis can be regarded as a progressive inflammatory process of the arterial wall ultimately leading to the formation of plaques. Currently, cardiovascular atherosclerotic disease is the leading cause of death in men and women in the western world (1). Atherosclerotic thickening of the vascular wall may compromise end-organ blood flow, but the greatest risk for clinical events is present in patients with an unstable plaque. Rupture of such a vulnerable plaque is one of the predominant underlying processes in the pathogenesis of both myocardial infarction and ischemic stroke. Histological assessment of ruptured plaques has shown several distinctive features, including a large lipid core composed of free cholesterol, oxidized lipids, a thin fibrous cap depleted of smooth muscle cells and collagen, inflammatory cell infiltration and increased adventitial and plaque neovascularization (2). Currently, indications for interventional treatment are based on clinical symptomatology and degree of stenosis (>70%) (3, 4). However, it has been recognized that plaque vulnerability rather than plaque size or severity of stenosis is important for plaque rupture risk assessment (5). In this context, the extent and location of plaque inflammation appear to be key factors in determining plaque vulnerability (6). In current clinical practice, anatomic imaging modalities, such as B-mode duplex ultrasound, computed tomography angiography scanning, and magnetic resonance imaging, can identify stenotic plaques of the internal carotid artery, but they give little or no information regarding molecular and cellular processes (5, 6).

Previous studies have shown that inflammation is related to the accumulation of activated macrophages at sites of plaque rupture (7). For this reason, the activated macrophage has emerged as an important diagnostic and therapeutic target for atherosclerosis (8, 9). The folate receptor β (FR-β) is present on activated macrophages but not on quiescent macrophages or other immune cells (10-12). Another family member, folate receptor α, is expressed on many epithelial cancers (13), while folate receptor γ is secreted by hematopoietic cells at levels generally too low to detect by standard methods (14). Folate receptor δ is restricted to regulatory T cells and for unknown reasons does not bind folate (15). In general, the folate receptor (FR) is a 38 kDA glycosylphospatidylinositol-anchored protein that binds to the vitamin folic acid with high affinity (KD<1nM) (12).

By visualizing the FR-β with an optical fluorescent contrast agent, activated macrophages can be detected by optical imaging. As such, van Dam et al. conjugated fluoresceine-isothiocynate (FITC) with folate (i.e. folate-FITC) to identify tumor processes, as several solid tumors express folate receptor α (16). Similarly,Ayala-López et al. showed a folate-^{99m}Tc-EC20 compound binds to the FR-β and therefore localizes folate in apolipoprotein E knock-out mice fed a western diet. (11).

Vulnerable plaques are also characterized by intra-plaque hypoxia (17). The transcription factor hypoxia-inducible factor-1 alpha (HIF-1α) is regarded as the major oxygen homeostasis regulator. During hypoxia, HIF-1α levels rapidly increase and dimerize to HIF-

1β to form the stable heterodimer transcription factor HIF-1. HIF-1 plays an important role in angiogenesis and is involved in tumor growth leading to resistance to radiotherapy and chemotherapy, as well as in inflammatory reactions (18, 19).
The aim of this pilot study was to explore the potential of fluorescence labeled folate as a macrophage activity marker in vessel wall. Thus we investigate whether areas of high folate-FITC uptake, reflect the presence of an increased number of activated macrophages, characteristic of an unstable plaque in the internal carotid artery. In addition, we investigated HIF-1α expression in atherosclerotic plaques, and compared this to the distribution of FR-β within the plaque in order to confirm the relationship between hypoxia and plaque vulnerability. These data will contribute to the development of optical imaging of the carotid artery in the near future that could be used as a non-invasive imaging method to detect and quantify the vulnerability of atherosclerotic plaques.

Materials and methods

Study Design

Between October 2008 and April 2009, carotid specimens were taken from a total of 20 patients. These patients underwent open carotid surgery in two hospitals (University Medical Center Groningen (UMCG), and Martini Hospital, Groningen, The Netherlands). From these patients atherosclerotic plaques were obtained by means of a carotid endarterectomy (CEA) of the internal carotid artery using standard techniques. Patients who underwent surgical treatment were symptomatic patients (i.e. with a history of cerebrovascular accident (CVA)), transient ischaemic attack (TIA) or amourosis fugax) who had a stenosis of 70-99% as detected by duplex ultrasound. Also asymptomatic patients with a stenosis of 80-99% were eligible for surgical treatment (UMCG only). Risk factors such as smoking status, obesity (body mass index, BMI), hyperlipidemia, hypertension and diabetes mellitus were recorded. Hypertension was defined as systolic arterial pressure above 140 mmHg and/or diastolic arterial pressure above 90 mmHg, or use of antihypertensive drugs, prescribed with the aim to reduce blood pressure (20). Hyperlipidemia was diagnosed if plasma total cholesterol exceeded 6.21 mmol/l, plasma LDL cholesterol exceeded 3.36 mmol/l, plasma triglycerides exceeded 2.26 mmol/l, or if the patient used lipid-lowering drugs (20). The study was approved by the Institutional Review Board (IRB) of each hospital and informed consent was obtained from all patients.

Carotid Endarterectomy Sample Collection And Timepath Of Study

The carotid samples were collected during CEA. The samples where immediately transported in a phosphate buffer on ice to the IVIS spectrum, with a transport time of 21±5 minutes at the UMCG hospital, and 50±14 minutes at the Martini hospital. After the samples were collected and transported, imaging with the IVIS spectrum was performed after determining the optimal incubation time and concentration (figure 2B). To validate

the folate-FITC signal, protein levels (immunohistochemistry, immunofluorescence) and gene-levels (RT-PCR) were measured.

Imaging Of The Plaque

Ex vivo fluorescence imaging (FLI) was performed to determine the exact location where the folate-FITC (MW 916 kDa, Supplemental figure 1) uptake had taken place within the plaque. Before imaging the concentration and incubation time were determined by making a serial dilution. Several concentrations were tested. Due to the highest intensity found at an incubation time of 30 minutes, in 2 ml of folate-FITC in PBS and a folate-FITC:PBS dilution of 1:10 (10 µg/ml), we considered this as the ideal concentration and incubation time. Fluorescence images were taken with a commercial imaging system with an ultra-sensitive charge-coupled device (CCD) camera mounted on a light-tight black chamber IVIS® Spectrum (Caliper Life Sciences, Hopkinton MA, U.S.A.). In this imaging system we used wavelengths of 500 nm excitation wave, and 540 nm emission wave for every sample. To suppress autofluorescence from surrounding tissue spectral unmixing algorithms were used. The technique of fluorescence imaging with the system has been described previously (*22*). Sections were taken at locations with high intensity (i.e. > $5.00e^{-4}$ radiance, photons/sec/cm^2/sr) folate-FITC uptake (so-called hot spots), medium spots (medium uptake, intensity $2.00e^{-4}$ till $5.00e^{-4}$ radiance, photons/sec/cm^2/sr) and cold spots (low uptake intensity < $2.00e^{-4}$ radiance, photons/sec/cm^2/sr). Cold spots served as negative control tissue. Images acquired were processed using Living Image version 3.0® software (Caliper Life Sciences, Hopkinton MA, U.S.A.).

Validation Of Folate-FITC Labeling

To investigate whether FR-β is present on activated macrophages and whether folate-FITC (kindly provided by prof. P.S. Low) binds to FR-β, we repeated the experiment as described by Xia et al.(10). Macrophages expressed in synovial fluid of rheumatoid arthritis patients are considered to be activated because proinflammatory or regulatory cytokines and growth factors, tumor necrosis factor (TNF)-α, granulocyte– macrophage colony-stimulating factor (GM-CSF), chemokines and chemoattractants, metalloproteinases and neopterin are all overexpressed (21). Synovial fluid was obtained from patients with rheumatoid arthritis, who had given informed consent, and mononuclear fraction was collected by Ficoll gradient separation. Cells were stained with anti-CD11b-PE (BD Biosciences, California, USA) to distinguish macrophages from other immune cells. To demonstrate presence of FR-β on the macrophages, the receptor was visualized using biotinylated anti-human FR-β antibody (R&D systems). Streptavidine-APC (Biolegend) was used as secondary antibody. These cells were also incubated with folate-FITC (100 nM, 0.05 µg/ml) and to test if this was specific binding we also incubated with 100-fold excess of free folic acid (nr. 1.03984, Merck, Darmstadt, Germany).

Immunohistochemistry

Biopsies were taken of the first 10 patients through the plaque at the sites of 'hot', 'medium' and 'cold' spots represented on the IVIS® spectrum. Subsequently these spots were checked with this system, by making a new fluorescence image to make sure this piece was a hot, medium or cold spot indeed. Next they were embedded in paraffin, and sections of 4 µm were made of all spots of 10 patients. After deparaffinization endogenous peroxidase activity was blocked by 0.3% H_2O_2 incubation for 30 minutes. Macrophages were identified using monoclonal mouse anti-human antibody CD68 antibody (1:50; clone PG-M1 DAKO, Glostrup, Denmark) for 60 minutes at room temperature. Endothelial cells were made visible by using mouse anti-human anti-CD31 monoclonal antibody (1:25) clone JC70A (DAKO) for 60 minutes at room temperature. To visualize areas of decreased oxygen level i.e. hypoxia HIF-1α (hypoxia-inducible factor -1α) stainings were performed with mouse antibody HIF-1α 67sup (Abcam, Cambridge, UK) overnight at 4°C (23). Labeled polymer-HRP anti mouse from the Dako K4006 kit were used as second antibody. Color reaction was developed using DAB staining with chromogen, and sections were counterstained with hematoxylin. Nuclei were therefore stained blue, and specific immunostaining was shown brown. Immunoreactivity in the plaques was scored by two independent observers as follows: (0 = none; 1 = mild; 2 = moderate; 3 = extensive) (24)

Immunofluorescence

To demonstrate co-localization of folate receptor β with macrophages, fluorescence microscopy with CD68 and FR-β was performed on hot spots and cold spots of atherosclerotic carotid plaque, determined with the IVIS® spectrum. After deparaffinization, antigen retrieval with 10mM tris-HCL + 1 mM EDTA was performed for 60 minutes. Subsequently endogenous peroxidase activity was blocked by 0.3% H_2O_2 incubation for 30 minutes. Macrophages were identified using the same antibody, concentration and incubation time as used with immunohistochemistry. FR-β was visualized using biotinylated anti-human FR-β antibody (1:10) overnight at 4°C (R&D systems). TRITC-goat anti-mouse IgG3 (Southern Biotech) and streptavidine-APC (Biolegend) were used as secondary antibodies respectively. DAPI staining was performed to stain nuclei. Pictures were taken using the Quantimet (Leica, 600S).

RNA Isolation, cDNA Synthesis And qRT-PCR

In this study, it appeared that RNA-isolation from the imaged plaques was not possible due to breakdown of RNA during folate-FITC incubation at room temperature. During the process of incubation for 30 minutes, RNA remained stable (and detectable) with rtPCR for only a few minutes. To investigate mRNA expression of relevant proteins in atherosclerotic plaques we used biopsies from atherosclerotic plaques from the carotid artery directly stored at -80 °C immediately after explantation, which had not been imaged in the IVIS Spectrum. RNA isolation, cDNA synthesis and qRT-PCR were performed. In short, TRIzol was added to

the frozen tissues, which were then ground with a pestle. Total RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). The reverse transcriptase step was performed using reagents from Invitrogen (Breda, the Netherlands). One µg of total RNA was transcribed to cDNA with 0.5 µl 7.5 µM Oligo (dT) 24 primers, 1 µl M-MLV Reverse Transcriptase (RT) enzyme, 5 x first strand buffer, 0.9 µl 0.1 mM DTT, 25 mM dNTP's in a total volume of 25 µl RNAse free water. For measurement of mRNA 1 µl cDNA sample was added in duplicate for amplification by the Taqman real time PCR system (ABI Prism 7900HT Sequence Detection system, Applied Biosystems, Foster City, CA, USA). Investigated genes were: GAPDH, CD68, CD31, HIF-1α, HIF-2α, VEGF (vascular endothelial growth factor), FR-α (folate receptor 1 or α), FR-β (folate receptor 2 or β), IL-8, MMP-9 (matrix metalloproteinase 9), Ang-1 (angiopoietin 1) and Ang-2 (angiopoietin 2). Ct (threshold cycle) values were determined with the software program SDS 2.3 (Applied Biosystems, Foster City, CA, USA). Samples that needed more then 30 cycles of GAPDH (reference gene) before reaching threshold were discarded. Relative gene expression was normalized to the expression of GAPDH and calculated by the following formula; Relative gene expression = $2^{-\Delta Ct}$ (ΔCt = Ct gene of interest – Ct GAPDH). Furthermore, samples of non-atherosclerotic umbilical artery were used as a control.

Statistics

Values are presented as mean ± standard deviation. For comparison of fluorescence efficiency measurements in the IVIS spectrum, a two-tailed, paired student T-test was used for parametric distributions. Non-paired continuous variables with a non-parametric distribution were analyzed using the Mann-Whitney U-test. For correlations, Pearson's and Spearman's correlation coefficients were used when appropriate. A two-sided P value < 0.05 was considered statistically significant. Statistical tests were done with the Statistical Package for the Social Sciences (SPSS statistics version 16.0, SPSS inc®, Chicago IL, USA).

Results

Patient Characteristics

A total of 17 men and three women with a mean age of 68.7 ± 9.4 years were included. Twenty carotid plaques were obtained during surgery. Patient characteristics are shown in Table 1.

Table 1. Baseline characteristics and risk factors for atherosclerosis

	Patients (n=20)
Men, n (%)	17 (85)
Age, years	69 ± 9
BMI, kg/m2	25 ± 5
Smoking status, n (%)	8 (40)
> 1 pack a day, n (%)	3 (38)
≤ 1 pack a day, n (%)	5 (62)
Hypertension, n (%)	18 (90)
Systolic blood pressure, mmHg	152 ± 21
Diastolic blood pressure, mmHg	83 ± 11
Use of antihypertensives, n (%)	18 (90)
Hyperlipidemia, n (%)	12 (60)
Use of lipid-lowering drugs, n (%)	19 (95)
LDL, mmol/L	3.2 ± 1.1
HDL, mmol/L	1.1 ± 0.4
Triglycerides, mmol/l	1.5 ± 0.7
Diabetes mellitus, n (%)	1 (5)

Unless otherwise indicated, data are expressed as mean ± standard deviation; percentages between brackets.

Fluorescence Imaging and Validation of Folate-FITC Labeling

To validate the folate-FITC signal, CD11b cells were stained for FR-β. In isolated mononuclear cells from synovial fluid of a rheumatoid arthritis patient a large population of macrophages was found (63% were CD11b positive cells). Staining CD11b positive cells for FR-β shows the majority of the activated macrophages express FR-β (figure 1). Staining with folate-FITC gives a high percentage of positive cells, which can be blocked with free folic acid indicating that this is a specific interaction between FR-β and folate-FITC .

Figure 1.

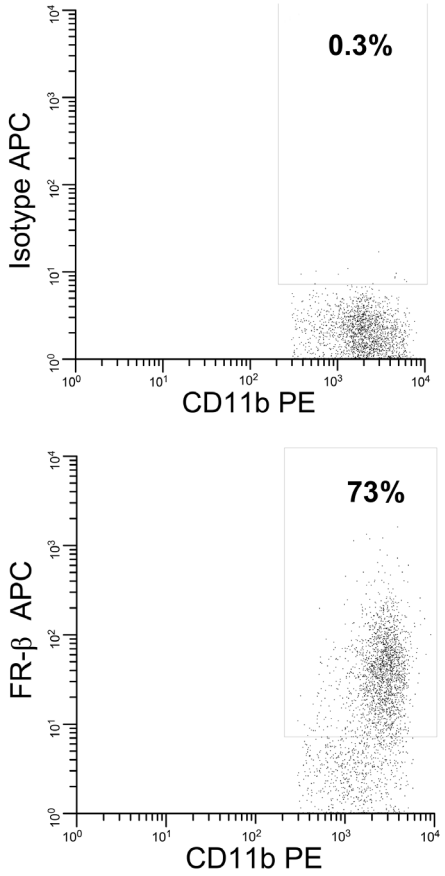


Figure 1. Flowcytometric analysis of macrophages from synovial fluid of a rheumatoid arthritis patient. Of this macrophage population, 73% express FR-β

During fluorescence imaging of the plaques, using the IVIS spectrum®, the fluorescence signals reflecting the folate-FITC uptake occurred in specific areas of the plaques (Figure 2A). Fluorescence signals increased after 30 minutes of incubation with folate-FITC and reached a plateau phase after 30 minutes (Figure 2B). When this plateau was reached, the final images for comparison were taken. There was a heterogeneous distribution of fluorescence signals across the plaque's surface, both at the intraluminal and extraluminal surfaces. Mean background fluorescence (radiance, photons/sec/cm^2/sr) as measured prior to incubation with folate-FITC was $1.5e^{-3} \pm 7.4e^{-4}$ intraluminally, and $2.5e^{-3} \pm 9.7e^{-4}$ extraluminally ($P<0.001$). After incubation with folate-FITC these values were $9.8e^{-3} \pm 2.6e^{-2}$ (intraluminally), and $5.5e^{-3} \pm 0.4e^{-3}$ (extraluminally) ($P<0.001$). Folate-FITC/background fluorescence ratios did not differ significantly between both sides of the plaque. There was a significant difference in intensity between background signals and folate-FITC intraluminally as well as extraluminally ($P< 0.001$, Figure 2C).

Figure 2.

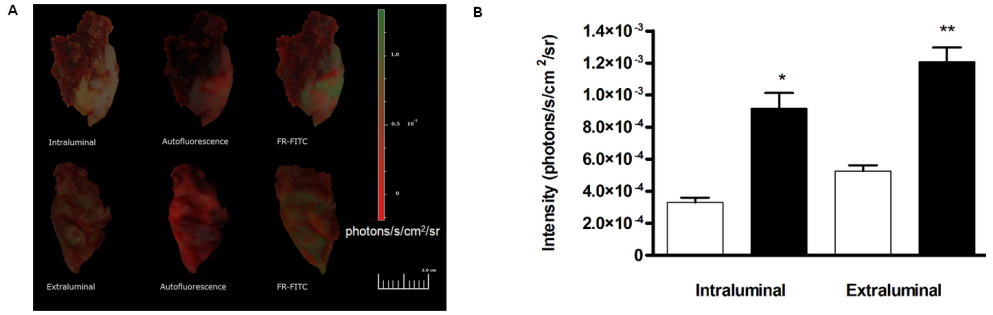


Figure 2. Ex vivo fluorescence imaging of human atherosclerotic plaques. (A) Fluorescence signals reflecting folate–FITC uptake occurring in specific areas of plaque intraluminally (upper) and extraluminally (bottom): incubation in phosphate-buffered saline (left), autofluorescence (middle), incubation with folate– FITC (right). (B) Intensity of fluorescence signals of plaques incubated with folate–FITC (solid bars) were increased both intraluminally and extraluminally when compared with autofluorescence signals (open bars). *P<0.001, **P<0.001. FR = folate receptor.

Immunohistochemistry and Immunofluorescence

Hot, medium and cold spots of 10 patients were stained with CD68 to identify macrophages in conjunction with folate-FITC uptake. Spots were identified as follows: cold spot exhibited low to no uptake; medium spot showed moderate uptake, and hot spot displayed high uptake of folate-FITC. Figure 3 shows a representative example of cold, medium and hot spots with respect to CD68 staining, along with quantification of the staining intensities at the cold, medium and hot spots.

Figure 3.

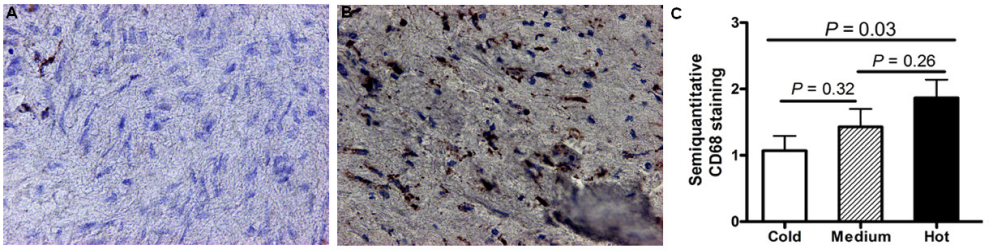


Figure 3. CD68 expression in atherosclerotic plaques of carotid arteries. (A) CD68 positive macrophages were nearly absent in a cold spot of such a plaque. (B) Extensive CD68 positive macrophage expression is present in a hot spot of a plaque. (C) The immunoreactivity score of CD68 was increased in hot spots (solid bar) versus cold spots (open bar) in 10 patients. (P=0.06)

In Figure 4 staining for HIF-1α in these identical spots is depicted. HIF-1α upregulation was significantly increased in the hot spot compared to the cold spot (P=0.01). As a control, expression of CD31-positive cells for the presence of endothelial cells was found heterogeneously on all plaque specimens and there was no difference between hot, medium or cold spots concerning expression of endothelial cells (P=0.89).

Figure 4.

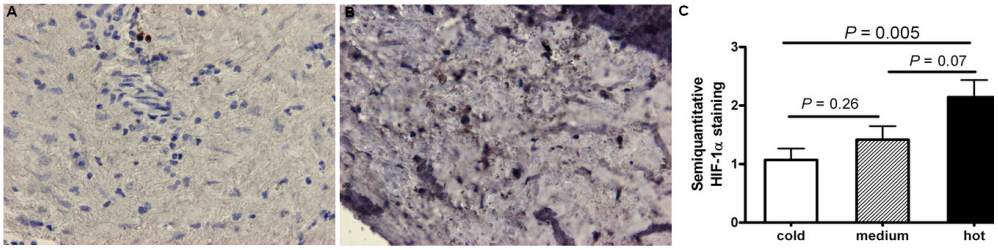


Figure 4. HIF-1α expression in atherosclerotic plaques of carotid arteries. (A) A cold spot of a plaque showed mild or no hypoxia (B) Hot spot of a plaque showing extensive hypoxia. (C) The immunoreactivity score of hypoxia was increased in hot spots (solid bar) versus cold spots (open bar) measured in 10 patients (P = 0.01).

To demonstrate co-localization of macrophages and FR-β in hot spots double staining using immunofluorescence with CD68 and FR-β was performed. Indeed, substantial co-localization of CD68 and FR-β expression was encountered, demonstrating the presence of FR-β on the majority of macrophages in hotspots (Figure 5).

Figure 5.

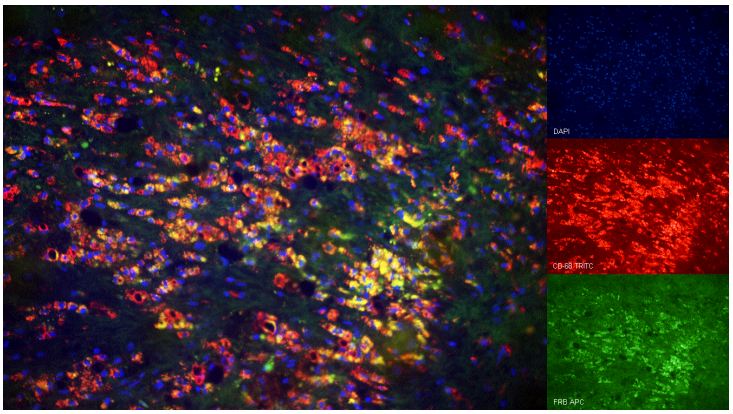


Figure 5. Colocalization of FR-β and macrophages in hot spot of plaque from carotid artery. Blue = DAPI staining of nuclei; red (TRITC) = CD68 expression, macrophage marker; green (APC) = FR-β expression. Overlay of yellow represents colocalization of CD68 and FR-β.

mRNA Expression

In 15 snap-frozen biopsies of atherosclerotic plaques and 3 control artery samples mRNA levels were determined of FR-α and -β, CD68 and CD31 (Figure 6A) and of HIF-1α, HIF-2α, VEGF, MMP-9, Ang-1, Ang-2, and IL-8 (Figure 6B). As expected, expression of the tumor-specific FR-α was not detected in the biopsies. Compared to control tissue mRNA expression of FR-β, CD68, and MMP-9 was significantly increased compared to control tissue (P=0.02, P=0.01, and P=0.01 respectively).

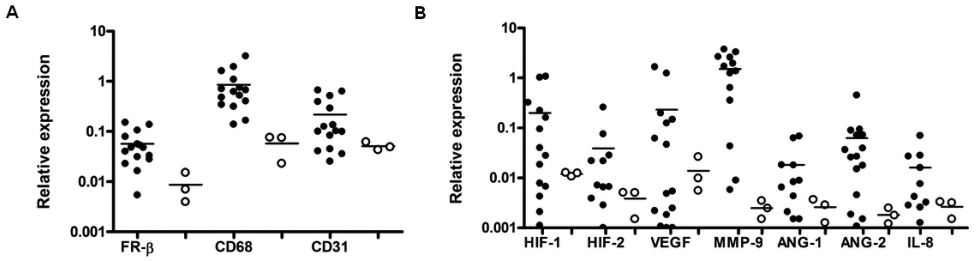


Figure 6.

Figure 6. Relative expression of mRNA levels in 15 atherosclerotic plaques and three control artery samples. Quantitative reverse transcription polymerase chain reaction was used to determine relative expression of different genes in human atherosclerotic plaques (black dots) compared to control artery tissue (open dots). (A) mRNA expression of FR-β and CD68 was significantly increased in atherosclerotic tissue compared to control tissue.(P=0.02 and 0.01 respectively) (B) mRNA expression of all inflammatory markers was higher in atherosclerotic tissue compared to control tissue.

FR-β expression moreover, significantly correlated with expression of the activated macrophage marker CD68 (r=0.53, P=0.045) (Figure 7). Also, CD68 expression showed a positive correlation with HIF-1α expression (r=0.55, P=0.034) and MMP-9 expression (r=0.63, P=0.021). CD31 expression was positively correlated with VEGF (r=0.66, P=0.007) and Ang-1 expression (r=0.63, P=0.039). No clear correlation could be found between FR-β and HIF1-α (r=0.07, P=0.13), nor between VEGF and Ang-1 (r=0.15, P=0.26).

Figure 7.

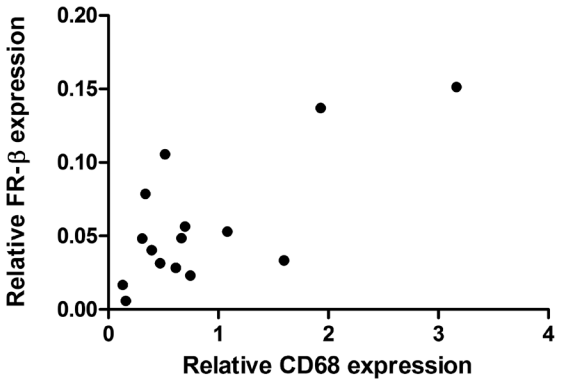


Figure 7. Correlation between mRNA levels of different genes in 15 human atherosclerotic plaques and three control artery samples. CD 68 expression versus FR-β expression determined by rt-PCR. A significant correlation was observed between CD68 expression and folate receptor-β expression (P=0.045).

Discussion

In this study we demonstrated high expression of folate receptor-β in plaques of patients with severe atherosclerotic disease using a novel methodology for mapping biomarker activity using optical folate-FITC imaging and confirmed our results histologically and by RT-PCR. Folate-FITC specifically binds to folate receptors and in this context to the FR-β, which is a marker for inflammation as is specifically expressed on activated macrophages (10, 12, 25). Moreover, the folate receptor-β on the cell surface of activated macrophages can bind folate-conjugated fluorophores with high affinity (12, 25). The significant difference between background fluorescence and folate-FITC uptake at both the intra- and extraluminal sides of the plaque might confirm the actual folate-FITC uptake in the plaque by ex-vivo incubation, which was found to be heterogeneously distributed. Furthermore, a difference between the number of activated macrophages in cold, medium and hot spots by folate-FITC guided mapping, respectively, could be detected, indicating increased inflammation in hot spots. However, this result was not significantly, which might be a result of the small group of patients used for this experiment (n= 10). In a previous study, an excess of macrophages over vascular smooth muscle cells was found in plaques vulnerable to rupture (26). In a study by Sluimer et al., the presence of macrophages correlated with hypoxia and levels of HIF-1α (24). Evidence of hypoxia in vascular disease is supported by in vivo detection of hypoxia in macrophage rich regions in rabbit atherosclerosis (6). These reports are supported by our findings on the HIF-1α stainings, which exhibited significant differential expression in the cold, medium, and hot spots, and correlated with the number of activated macrophages. This new approach of macrophage detection by folate-FITC labeling seems feasible to detect activated macrophages by their increased expression of folate receptor-β and is supported by double staining experiments (Figure 5). In addition to morphological and functional (duplex ultrasound) imaging, imaging of vulnerable plaques by means of activated macrophages might contribute to a better risk assessment and selection of patients at risk for ischemic stroke.

Activated macrophages have FR-β on their surface and produce MMP-9. Symptomatic plaques showed a high relative gene expression of MMP-9 (Figure 6B) and correlated with CD68 expression. This is in accordance with previous research, in which over-expression of MMP-9 in plaques had a strong association with unstable plaques, and with the presence of macrophages within these plaques (20, 27). Furthermore, analysis of the presence of MMP-9 protein by ELISA within excised carotid plaques revealed high MMP-9 protein mass in calcified segments at or near the carotid bifurcation and in segments with intraplaque hemorrhage (22). Since MMP-9 also can be visualized using the IVIS technique as shown earlier by our group, this could be combined with dual visualization of MMP-9 and FR-β, leading to a better assessment of inflammatory sites in vulnerable atherosclerotic plaques (22).

Endothelial cells produce VEGF and angiopoietins. The Ang-2 expression in the plaques

was relatively high compared to Ang-1. This is in agreement with previous findings, where Ang-1 induced formation of stable vessels, while Ang-2 destabilized the interaction between endothelial cells and their supporting cells (28). In the current study, CD31 correlated positively with VEGF and angiopoietin expression. As was expected, CD31 did not correspond with the folate receptor-β imaging since endothelial cells do not express folate receptors (Low, personal observations).

Fluorescence imaging using a folate-FITC optical contrast agent probe can visualize the distribution of activated macrophages, and might therefore be a good marker of inflammation and vulnerability of human atherosclerotic plaques. However, there are several limitations to this study. The number of carotid plaques was relatively small. Moreover, the IVIS Spectrum equipment used in the current ex vivo set-up cannot be applied for in vivo use in humans. FITc was used because this is currently approved for intravenous use in targeting the folate receptor alpha as a potential useful probe in oncology patients. Current applications for non-invasive optical imaging of fluorescent signals within a human carotid artery are limited by the use of fluorophores with emission wavelengths within the range of 450 tot 600 nm such as FITC, leading to a limited penetration depth. Therefore, sophisticated imaging devices such as multispectral optoacoustic tomography (MSOT) for imaging of near-infrared fluorophores like IRDye-CW800 conjugated to folate might solve this problem for future clinical applications. It is anticipated that such a device could also be used for clinical purposes for identification of high-risk vulnerable atherosclerotic plaques (29). We recently started with folate-^{99m}Tc (kindly provided by prof. P.S. Low) imaging. The results look promising; therefore we believe folate-^{99m}Tc might be a good compound for in vivo use in the future using the SPECT modality.

Furthermore, fluorescence imaging with the IVIS spectrum as it is used in the current set-up by definition is two-dimensional, i.e. a hot spot on the extraluminal side is not per se a hot spot on the intraluminal side. Future ex vivo 3D rendering and more sensitive quantification of picomolar concentrations using fluorescence molecular tomography (FMT), as described by Ntziachristos et al. might solve this problem and is currently under investigation (30).

In conclusion, high FR-β expression, as visualized by optical fluorescence imaging using folate-FITC, correlated with increased HIF-1α staining and a trend could be seen between high FR-β expression and CD68, representing activated macrophages. In addition, FR-β mRNA levels correlated with CD68, HIF-1α, and MMP-9 mRNA expression. Molecular imaging of the pathogenesis related to the vulnerability of atherosclerotic plaques might enable the clinician to make more substantiated therapeutic decisions in the treatment of patients with carotid artery disease in the future.

References

(1) Lindholm LH, Mendis S. Prevention of cardiovascular disease in developing countries. Lancet 2007 Sep 1;370(9589):720-722.
(2) Antohe F. Endothelial cells and macrophages, partners in atherosclerotic plaque progression. Arch Physiol Biochem 2006 Oct-Dec;112(4-5):245-253.
(3) Ringleb PA, Chatellier G, Hacke W, Favre JP, Bartoli JM, Eckstein HH, et al. Safety of endovascular treatment of carotid artery stenosis compared with surgical treatment: a meta-analysis. J Vasc Surg 2008 Feb;47(2):350-355.
(4) Zeebregts CJ, Meerwaldt R, Geelkerken RH. Carotid artery stenting: a 2009 update. Curr Opin Cardiol 2009 Nov;24(6):528-531.
(5) Wallis de Vries BM, van Dam GM, Tio RA, Hillebrands JL, Slart RH, Zeebregts CJ. Current imaging modalities to visualize vulnerability within the atherosclerotic carotid plaque. J Vasc Surg 2008 Dec;48(6):1620-1629.
(6) Nighoghossian N, Derex L, Douek P. The vulnerable carotid artery plaque: current imaging methods and new perspectives. Stroke 2005 Dec;36(12):2764-2772.
(7) Fuster V. Lewis A. Conner Memorial Lecture. Mechanisms leading to myocardial infarction: insights from studies of vascular biology. Circulation 1994 Oct;90(4):2126-2146.
(8) Tiwari RL, Singh V, Barthwal MK. Macrophages: an elusive yet emerging therapeutic target of atherosclerosis. Med Res Rev 2008 Jul;28(4):483-544.
(9) Choudhury RP, Lee JM, Greaves DR. Mechanisms of disease: macrophage-derived foam cells emerging as therapeutic targets in atherosclerosis. Nat Clin Pract Cardiovasc Med 2005 Jun;2(6):309-315.
(10) Xia W, Hilgenbrink AR, Matteson EL, Lockwood MB, Cheng JX, Low PS. A functional folate receptor is induced during macrophage activation and can be used to target drugs to activated macrophages. Blood 2009 Jan 8;113(2):438-446.
(11) Ayala-Lopez W, Xia W, Varghese B, Low PS. Imaging of atherosclerosis in apoliprotein e knockout mice: targeting of a folate-conjugated radiopharmaceutical to activated macrophages. J Nucl Med 2010 May;51(5):768-774.
(12) Paulos CM, Turk MJ, Breur GJ, Low PS. Folate receptor-mediated targeting of therapeutic and imaging agents to activated macrophages in rheumatoid arthritis. Adv Drug Deliv Rev 2004 Apr 29;56(8):1205-1217.
(13) Parker N, Turk MJ, Westrick E, Lewis JD, Low PS, Leamon CP. Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. Anal Biochem 2005 Mar 15;338(2):284-293.
(14) Elnakat H, Ratnam M. Distribution, functionality and gene regulation of folate receptor isoforms: implications in targeted therapy. Adv Drug Deliv Rev 2004 Apr 29;56(8):1067-1084.
(15) Yamaguchi T, Hirota K, Nagahama K, Ohkawa K, Takahashi T, Nomura T, et al. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. Immunity 2007 Jul;27(1):145-159.
(16) van Dam GM, Themelis G, Crane LM, Harlaar NJ, Pleijhuis RG, Kelder W, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-alpha targeting: first in-human results. Nat Med 2011 Sep 18.
(17) Luque A, Turu M, Juan-Babot O, Cardona P, Font A, Carvajal A, et al. Overexpression of hypoxia/inflammatory markers in atherosclerotic carotid plaques. Front Biosci 2008 May 1;13:6483-6490.
(18) Vink A, Schoneveld AH, Lamers D, Houben AJ, van der Groep P, van Diest PJ, et al. HIF-1 alpha expression is associated with an atheromatous inflammatory plaque phenotype and upregulated in activated macrophages. Atherosclerosis 2007 Dec;195(2):e69-75.
(19) Westra J, Molema G, Kallenberg CG. Hypoxia-inducible factor-1 as regulator of angiogenesis in

rheumatoid arthritis - therapeutic implications. Curr Med Chem 2010;17(3):254-263.
(20) Brevetti G, Silvestro A, Schiano V, Chiariello M. Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index. Circulation 2003 Oct 28;108(17):2093-2098.
(21) Wallis de Vries BM, Hillebrands JL, van Dam GM, Tio RA, de Jong JS, Slart RH, et al. Images in cardiovascular medicine. Multispectral near-infrared fluorescence molecular imaging of matrix metalloproteinases in a human carotid plaque using a matrix-degrading metalloproteinase-sensitive activatable fluorescent probe. Circulation 2009 May 26;119(20):e534-6.
(22) Kinne RW, Brauer R, Stuhlmuller B, Palombo-Kinne E, Burmester GR. Macrophages in rheumatoid arthritis. Arthritis Res 2000;2(3):189-202.
(23) Brouwer E, Gouw AS, Posthumus MD, van Leeuwen MA, Boerboom AL, Bijzet J, et al. Hypoxia inducible factor-1-alpha (HIF-1alpha) is related to both angiogenesis and inflammation in rheumatoid arthritis. Clin Exp Rheumatol 2009 Nov-Dec;27(6):945-951.
(24) Sluimer JC, Gasc JM, van Wanroij JL, Kisters N, Groeneweg M, Sollewijn Gelpke MD, et al. Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. J Am Coll Cardiol 2008 Apr 1;51(13):1258-1265.
(25) Muller C, Forrer F, Schibli R, Krenning EP, de Jong M. SPECT study of folate receptor-positive malignant and normal tissues in mice using a novel 99mTc-radiofolate. J Nucl Med 2008 Feb;49(2):310-317.
(26) Newby AC, George SJ, Ismail Y, Johnson JL, Sala-Newby GB, Thomas AC. Vulnerable atherosclerotic plaque metalloproteinases and foam cell phenotypes. Thromb Haemost 2009 Jun;101(6):1006-1011.
(27) Alvarez B, Ruiz C, Chacon P, Alvarez-Sabin J, Matas M. Serum values of metalloproteinase-2 and metalloproteinase-9 as related to unstable plaque and inflammatory cells in patients with greater than 70% carotid artery stenosis. J Vasc Surg 2004 Sep;40(3):469-475.
(28) Post S, Peeters W, Busser E, Lamers D, Sluijter JP, Goumans MJ, et al. Balance between angiopoietin-1 and angiopoietin-2 is in favor of angiopoietin-2 in atherosclerotic plaques with high microvessel density. J Vasc Res 2008;45(3):244-250.
(29) Razansky D, Harlaar NJ, Hillebrands JL, Taruttis A, Herzog E, Zeebregts CJ, et al. Multispectral Optoacoustic Tomography of Matrix Metalloproteinase Activity in Vulnerable Human Carotid Plaques. Mol Imaging Biol 2011 Jul 1.
(30) Ntziachristos V, Razansky D. Molecular imaging by means of multispectral optoacoustic tomography (MSOT). Chem Rev 2010 May 12;110(5):2783-2794.

CHAPTER 7

Folate receptor-β imaging using 99mTc-folate to explore distribution of polarized macrophage populations in human atherosclerotic plaque

Nynke A. Jager, Johanna Westra, Reza Golestani, Gooitzen M. van Dam, Philip S. Low, René A. Tio, Riemer H.J.A. Slart, Hendrikus H. Boersma, Marc Bijl, Clark J. Zeebregts

J Nucl Med. (accepted)

Abstract

OBJECTIVES In atherosclerotic plaques the risk of rupture is increased at sites of macrophage accumulation. Activated macrophages express folate receptor-β (FR-β) which can be targeted by folate coupled to radioactive ligands to visualize vulnerability. The aim of this study is to explore presence of activated macrophages in human atherosclerotic plaques by ^{99m}Tc-folate imaging and to evaluate whether this technique can discriminate between a M1-like and M2-like macrophage phenotype.

METHODS Carotid endarterectomy specimens of 20 patients were incubated with ^{99m}Tc-folate, imaged using μSPECT, and divided in 3mm slices. Mean accumulation was calculated per slice and distribution of M1-like and M2-like macrophages per slice was quantified by immunohistochemical staining for CD86 and iNOS for M1, and CD163 and FR-β for M2 macrophages. Monocytes from healthy donors were differentiated towards M1-like or M2-like phenotype by *in vitro* culturing. mRNA levels of specific M1- and M2-markers were measured by RT-PCR and expression of FR-β, CD86, and CD163 by flow cytometry.

RESULTS There was a heterogeneous accumulation of ^{99m}Tc-folate in plaques (median 2.45 (0.77-6.40) MBq/g). Slices with highest ^{99m}Tc-folate accumulation of each plaque showed significantly more expression of FR-β and CD163, compared to slices with lowest ^{99m}Tc-folate accumulation which showed significantly more expression of iNOS. In *in vitro* polarized macrophages, mRNA expression of FR-β, mannose receptor, IL-10 and MMP-9 was significantly increased in M2-like macrophages compared to M1-like macrophages. On receptor level CD86 was shown to be over expressed on M1-like macrophages while FR-β and CD163 where over expressed on M2-like macrophages measured by flow cytometry.

CONCLUSION In areas of high ^{99m}Tc-folate accumulation, more M2-like macrophages were present compared to areas with low accumulation. It is anticipated that ^{99m}Tc-folate imaging using SPECT as a marker for M2-like macrophages in atherosclerosis might be a good indicator for plaque vulnerability.

Keywords Carotid artery; atherosclerotic plaque; vulnerability; folate receptor-β imaging; M2-like macrophages.

Introduction

Cardiovascular atherosclerotic disease is the main cause of death in the Western world (1). Clinical events such as myocardial infarction and ischemic cerebrovascular accident are directly related to the risk of atherosclerotic plaque rupture. Histological analysis of a vulnerable plaque suggests the importance of necrotic core size, extent of inflammation, and angiogenesis, lipid accumulation, and fibrous cap thickness (2). Furthermore, in plaque vulnerability matrix metalloproteinase's (MMPs), reactive oxygen species, inflammatory cytokines and various growth factors released by activated macrophages play important roles (3). Patients with symptoms and carotid artery stenosis >70% or those asymptomatic with carotid artery stenosis of 80-90%, are deemed suitable for endarterectomy thereby significantly reducing the risk of subsequent major cerebrovascular accident or death (4). Imaging techniques currently used to visualize carotid artery stenosis include duplex ultrasound, computed tomography angiography (CTA) and angiography (5). However, these techniques focus on anatomic features of the plaque and barely give any information on molecular and cellular processes. They therefore cannot distinguish between stable and unstable plaques. As plaque composition rather than stenosis is important in detecting an unstable plaque, new imaging modalities are needed (5).

2-deoxy-2-[^{18}F]fluoro-D-glucose positron emission tomography ([^{18}F]FDG-PET) can be used to identify vulnerable plaques in atherosclerotic disease, since FDG accumulates in activated macrophages. FDG-microPET accumulation correlated well with macrophage content in a rabbit model, while in vivo quantification did not support this finding, possibly due to high accumulation in surrounding tissue (6). Folate receptor β (FR-β) is present on activated macrophages, but not on resting macrophages or other immune cells. For that reason it might be a good indicator for inflammation in the human atherosclerotic carotid plaque, as previously shown by imaging carotid artery specimens with fluorescence labeled folate (7). Furthermore, Ayala-López et al. localized activated macrophages in apolipoprotein E knockout mice fed a western diet using a ^{99m}Tc-folate labeled compound (8).

A heterogeneous population of macrophages exists including a classically activated macrophage type (M1) as well as an alternatively activated macrophage population (M2) (9). The M1 macrophage is thought to have pro-inflammatory properties and polarization in vitro is driven by interferon gamma and low concentration lipopolysacharride. Macrophages are driven towards the M2 type when the environment includes IL-4 and IL-10 (10). However, polarization of macrophages within a plaque is determined by the local micro-environment present in the atherosclerotic lesion and is thought to be rather more complex than the often used M1/M2 paradigm (11). As MMPs trigger plaque rupture, a M2-like macrophage type might be important as a direct cause of plaque vulnerability by producing tissue degrading MMPs (12).

Therefore, the major goal of this pilot study was to explore the distribution of macrophage

subtypes in atherosclerotic plaques and the FR-β expression on the respective macrophage subtypes. In addition, the potential of technetium labeled folate as marker for atherosclerotic carotid plaque vulnerability was explored. To achieve this goal, the ^{99m}Tc-folate signal was compared with FR-β staining using specimens with a positive imaging ratio (accumulation slice/total plaque accumulation>1) to validate the folate technetium compound. Additionally, macrophages were differentiated and polarized in vitro into M1-like and M2-like macrophages to investigate expression of FR-β and known M1 and M2 markers.

Materials and Methods

Study Design

Between August 2011 and May 2012, a total of 20 carotid specimens were obtained by means of carotid endarterectomy (CEA) of the carotid bifurcation using standard techniques (13). Patients underwent open carotid surgery at the University Medical Center Groningen (UMCG) and were symptomatic (i.e. with a history of recent cerebrovascular accident (CVA)), transient ischaemic attack (TIA) or amaurosis fugax). They presented with a stenosis of the common carotid artery of 70-99% as detected by duplex ultrasound. Additionally, asymptomatic patients with a stenosis of 80-99%, found by routine control were eligible for surgical treatment. Risk factors such as hyperlipidemia, hypertension, smoking status, obesity (body mass index, BMI) and diabetes mellitus were recorded. Hyperlipidemia and hypertension was defined as described before by our group (7). For validation of macrophage markers six healthy volunteers where included without known cardiovascular disease or risk factors. The study was approved by the Institutional Review Board (IRB) of the UMCG and informed consent was obtained from all patients and healthy volunteers.

Carotid Endarterectomy Sample Collection and Timepath of Study

The carotid samples collected during CEA, were immediately transported in a phosphate buffer on ice to the U-SPECT-II system (MILabs, Utrecht, The Netherlands), with a mean transport time of 18±6 minutes. The optimal incubation time and concentration of ^{99m}Tc-folate was determined. To validate the ^{99m}Tc-folate signal, immunohistochemistry was used for determining M1-like and M2-like macrophage levels in regions with high and low ^{99m}Tc-folate accumulation.

^{99m}Tc-folate Labeling and Imaging of the Plaque using μSPECT

To determine the exact location where the ^{99m}Tc-folate accumulation had taken place within the plaque, ex vivo imaging was performed. A ^{99m}Tc-labeled imaging agent consisting of the vitamin folic acid was conjugated to a chelating agent with specificity for ^{99m}Tc was used as follows; 1mL of Sodium Pertechnetate ^{99m}Tc injection (100 MBq) was injected into a

shielded vial. The vial was gently swirled for 30 seconds to dissolve the lyophilized powder and placed into a lead shield and incubated at 100 °C for 18 minutes. Subsequently, the ^{99m}Tc-folate vial was cooled for 15 minutes protected from light, and the radiochemical purity of ^{99m}Tc-folate was determined by ITLC using saline as the eluent. The radiochemical purity must be >90% to pass acceptance criteria. The carotid specimens were weighed and incubated for one hour in 5mL of a ^{99m}Tc-folate containing solution at room temperature. After washing ^{99m}Tc-folate accumulation was measured using the dose calibrator. The specimens were put into a micro single-photon emission computed tomography (μSPECT) scanner, near the center of field of view (FOV) and scanned for one hour (as determined by serial dilution). μSPECT images were corrected for scatter and reconstructed applying an interactive reconstruction algorithm (OSEM 2D). After incubation and scanning, all specimens were divided in equal slices of 3mm. For every slice the mean accumulation was calculated as counts/voxel of Region of Interest (ROI) versus total dose calibrator activity per gram (total plaque weight), expressed as MBq/g. Images acquired were processed using Amide software (14).

Immunohistochemistry and Validation of ^{99m}Tc-folate Signal

After determining ^{99m}Tc-folate accumulation of every slice, slices of plaques of 10 patients were embedded in paraffin, and sections of 4 μm were made. Macrophages were identified by incubation with monoclonal mouse anti-human CD68 (1:50; mo876 clone PG-M1 DAKO, Glostrup, Denmark). For detection of M1 macrophages, rabbit anti-human iNOS and rabbit anti-human antibody CD86 (ab15323 and ab53004 respectively, Abcam, Cambridge, UK) were used. To show M2-like macrophage distribution mouse anti-human CD163 (a widely used M2 macrophage marker and mainly found in advanced human lesions near intraplaque haemorrhage areas (15), ab74604, Abcam, Cambridge, UK) and biotinylated sheep anti-human FR-β antibody were used (BAF 5697, R&D systems, Minneapolis, USA). Also, FR-β and CD163 were used for double staining to show co-localization of M2-like macrophages and FR-β expression. Appropriate secondary antibodies labeled with horseradish peroxidase (HRP), and alkaline phosphatase (AF) for double staining was used. Color reaction was developed using DAB (Dako) staining with chromogen (fast red (Sigma) for double staining with AF), and sections were counterstained with hematoxylin. All sections were stored digitally after examination using a Nanozoomer Digital Pathology Scanner (NDP Scan U10074-01, Hamamatsu Photonics K.K., Japan) and quantified (percentage of positive cells / total cells) with software of ImageScope Viewer (V11.2.0.780 Aperio, e-Pathology Solution, CA, USA). For validation, slices with the lowest mean accumulation of the ^{99m}Tc-folate signal in imaging where compared with the slices with the highest mean accumulation in every carotid plaque separately (both compared to mean accumulation in the total plaque in counts/voxel).

In Vitro Differentiation and Polarization of M1-like and M2-like Macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from 6 healthy donors by density gradient centrifugation using Lymphoprep (Axis Shield PoC As, Oslo, Norway). Subsequently, monocytes were allowed to adhere to culture plates. The adherent cells were maintained for 5 days in RPMI 1640 medium (Lonza, Walkersville, MD, USA) supplemented with 10% filtered fetal calf serum (FCS). 20 ng/ml macrophage colony stimulating factor (M-CSF, R&D systems) was added to the cell culture for differentiation into macrophages. Macrophages were directed towards M1-like and M2-like phenotypes by use of 48 hours stimulation with 100 U/ml interferon gamma (PeproTech, USA) and 1 ng/ml lipopolysacharride (Sigma, Germany) or 20 ng/ml IL-4 and 10ng/ml IL-10 (PeproTech), respectively.

Flow Cytometry of M1-like and M2-like Macrophages

In vitro differentiated M1-like and M2-like macrophages were stained with biotinylated antihuman FR-β antibody (R&D systems) to explore protein expression. Streptavidine–allophycocyanine (APC, Biolegend, San Diego, CA, USA) was used as secondary antibody. Anti-CD86-FITC (BD Bioscience, San Jose, CA, USA) and anti-CD163-PE (Biolegend) were used to distinguish expression of these markers on macrophage subtypes. These were measured using flow cytometry.

RNA Isolation, cDNA Synthesis and qRT-PCR

RNA was extracted from M1-like and M2-like macrophages as described before (7). For measurement of mRNA (messenger ribonucleic acid) 1 µl cDNA sample was added in duplicate for amplification by the Taqman real time PCR system (ABI Prism 7900HT Sequence Detection system, Applied Biosystems, Foster City, CA, USA). Investigated genes were: GAPDH, IL-6, TNF, Toll-like receptor (TLR) 2 and 4, FR-β (folate receptor 2 or β), mannose receptor (CD206 or MR), IL-10 and MMP-9 (matrix metalloproteinase 9). Ct (threshold cycle) values were determined using the software program SDS 2.4 (Applied Biosystems). Relative gene expression was normalized to the expression of GAPDH and calculated by the following formula; Relative gene expression = $2^{-\Delta Ct}$ (ΔCt = Ct gene of interest – Ct GAPDH).

Statistics

Values are presented as mean ± standard deviation or median (range), unless stated otherwise. For correlations, Pearson's and Spearman's correlation coefficients were used when appropriate. A two-tailed, paired student T-test was used for parametric distributions. Non-paired continuous variables with a non-parametric distribution were analyzed using the Mann-Whitney U-test. A two-sided P value < 0.05 was considered statistically significant. Statistical tests were done with the Statistical Package for the Social Sciences (SPSS statistics version 20.0, SPSS inc®, Chicago IL, USA).

Results

Patient Characteristics

A total of 12 men and eight women with a mean age of 68.0 ± 8.4 years were included. Patient characteristics are shown in Table 1.

Table 1. Baseline characteristics and risk factors for atherosclerosis

	Patients (n=20)
Men, n (%)	12 (60)
Age, years	68 ± 8
Symptomatic, n (%)	18 (90)
Transient ischemic attack (TIA), n (%)	8 (40)
Cerebro vascular accident (CVA), n (%)	7 (35)
Amaurosis fugax, n (%)	3 (15)
BMI, kg/m2	27 ± 4
Smoking status, n (%)	10 (50)
> 1 pack a day, n (%)	4 (20)
≤ 1 pack a day, n (%)	6 (30)
None, smoked in last 10 years (%)	3 (15)
Hypertension, n (%)	15 (75)
Controlled with single drug, n (%)	6 (30)
Controlled with 2 drugs, n (%)	5 (25)
Requires > 2 drugs or uncontrolled, n (%)	4 (20)
Systolic blood pressure, mmHg	138 ± 15
Diastolic blood pressure, mmHg	74 ± 8
Hyperlipidemia, n (%)	12 (60)
Use of lipid-lowering drugs, n (%)	17 (85)
Diabetes mellitus, n (%)	3 (15)*

* Two patients had diabetes controlled by diet or oral agents, one patient was on insulin. Data are expressed as mean ± standard deviation; percentages between brackets.

Imaging of the Plaque using μSPECT

Imaging of 20 atherosclerotic plaques using μSPECT showed ^{99m}Tc-folate accumulation occurred in specific areas of the plaques, with a median total activity of 2.45 (0.77-6.40) MBq/g measured in a dose calibrator (figure 1). After scanning all specimens were divided in equal slices and ratio per slice was calculated (accumulation slice/total plaque accumulation). There were 5 ± 1 slices per atherosclerotic carotid plaque. ^{99m}Tc-folate accumulation was heterogeneously distributed throughout plaques (slices with highest ^{99m}Tc-folate ratio: 1.28 (1.03-2.24) and slices with the lowest ^{99m}Tc-folate ratio: 0.8 (0.18-0.99)).

Figure 1.

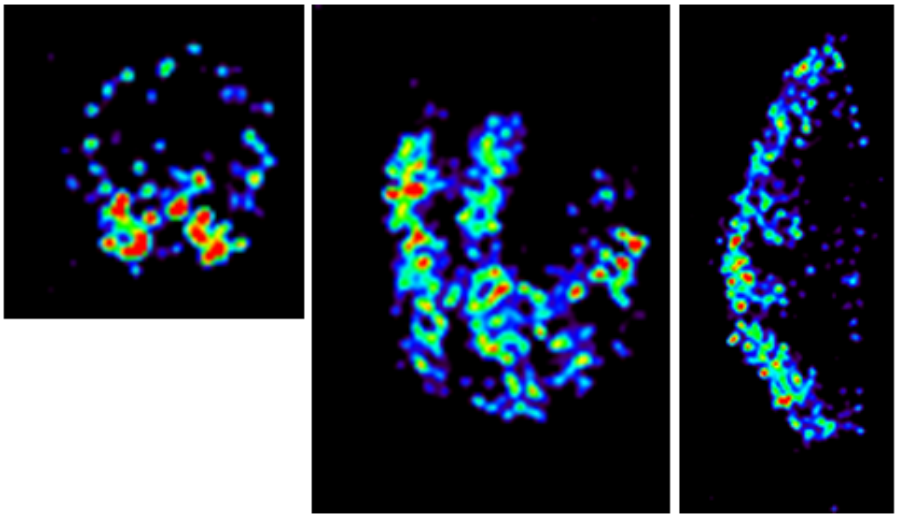


Figure 1. Transversal (left), coronal (middle), and sagittal (right) sections of microSPECT image of an atherosclerotic carotid plaque specimen after ^{99m}Tc-folate incubation showing heterogeneous accumulation. The transversal image shows that area of higher accumulation (red) is confined within the vessel wall.

Immunohistochemistry and Validation of ^{99m}Tc-folate Signal

Plaque slices of 10 patients were stained using FR-β to validate the ^{99m}Tc-folate signal, as well as for CD68 (pan macrophages), CD86 and iNOS (M1 macrophage markers) and CD163 (M2 macrophage marker). In figure 2A representative staining is shown. All stainings were quantified with Imagescope software and expressed as percentage of positive cells per total cells. Slices with a positive ^{99m}Tc-folate imaging ratio (accumulation slice/total plaque accumulation >1) where positively correlated to FR-β staining (P =0.006, figure 2B).

Figure 2.

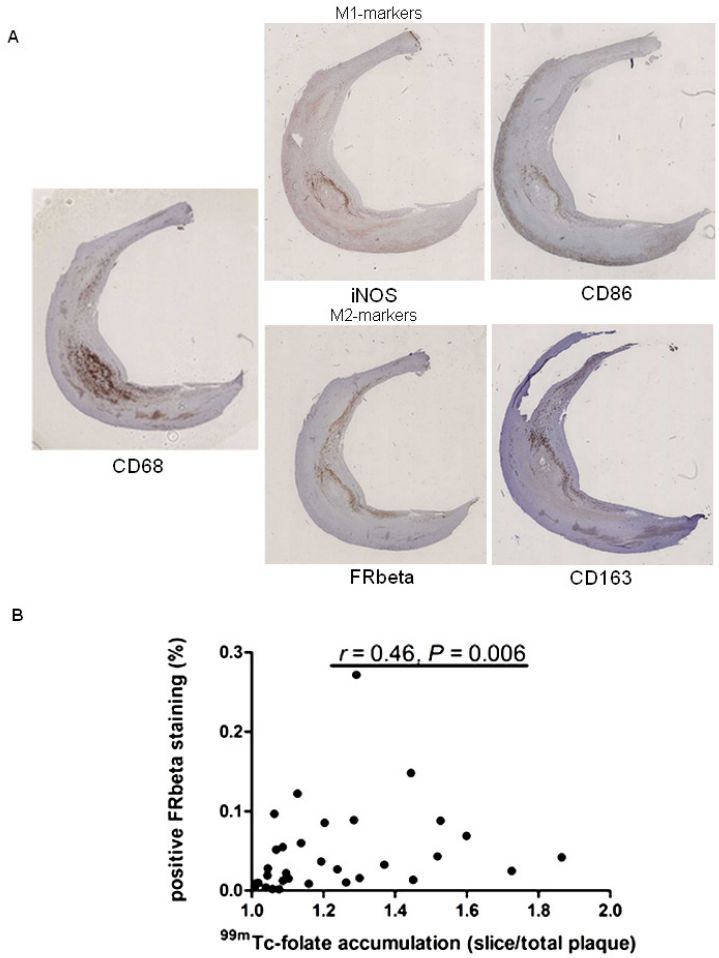


Figure 2. Immunohistochemical staining of 10 atherosclerotic plaques. (A) Staining of CD68, CD86, CD163, FR-β and iNOS is depicted in a slice with the highest accumulation of ^{99m}Tc-folate in a plaque. (B) Positive ^{99m}Tc-folate slices (accumulation slice / accumulation total plaque >1) showed a positive correlation with FR-β staining (r=0.46, P =0.006).

Figure 3 shows that FR-β staining in slices with the highest ^{99m}Tc-folate accumulation of each plaque was higher compared to slices with lowest accumulation (P=0.042). The same was shown for CD163 staining (P=0.036). iNOS staining, on the contrary, showed a significantly higher percentage of positive cells in the slices with lowest accumulation ^{99m}Tc-folate compared to slices with highest accumulation (P=0.043). However, staining of CD68 and CD86 was not significantly different in slices with highest accumulation ^{99m}Tc-folate compared to slices with lowest accumulation (P= 0.43 and P= 0.44 respectively). Furthermore, CD163 co localized with FR-β expression in a carotid plaque, as can be seen in a representative picture (Figure 3B).

Figure 3.

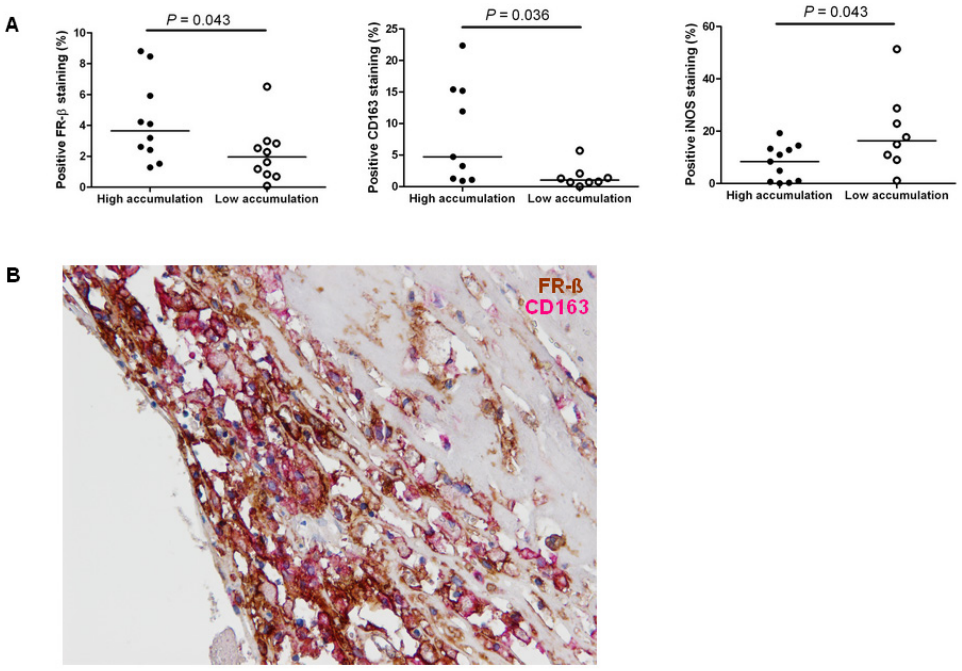


Figure 3 (A) Quantification of stainings (left) Slices with highest ^{99m}Tc-folate accumulation (closed dots) showed significantly more FR-β expression compared to slices with lowest ^{99m}Tc-folate accumulation (open dots, P = 0.043) (middle) Slices with highest ^{99m}Tc-folate accumulation (closed dots) showed significantly more CD163 staining compared to slices with lowest ^{99m}Tc-folate accumulation (open dots, P = 0.036). (Right) Slices with lowest ^{99m}Tc-folate accumulation (open dots) showed significantly more iNOS staining compared to slices with highest ^{99m}Tc-folate accumulation (closed dots, P = 0.043) (B) Double staining of CD163 and FR-β to show co-localization of M2-like macrophages and FR-β expression.

7

Expression of Macrophage Markers by Flow Cytometry

To validate FR-β, CD86 and CD163 as macrophage subtype markers, their expression on macrophage subpopulations was determined using flow cytometry (representative examples in figure 4). FR-β is expressed on 31 % of M1-like macrophages and on 72 % of M2-like macrophages. In M1-like macrophages, 93% (60-97) of cells were positive for CD86, while 34% (5-45) was positive for CD163. On the contrary, M2-like macrophages showed a high expression of CD163 (86% (56-95)) while CD86 expression was lower (27% (10-40)), both measured in 10 healthy volunteers.

Figure 4.

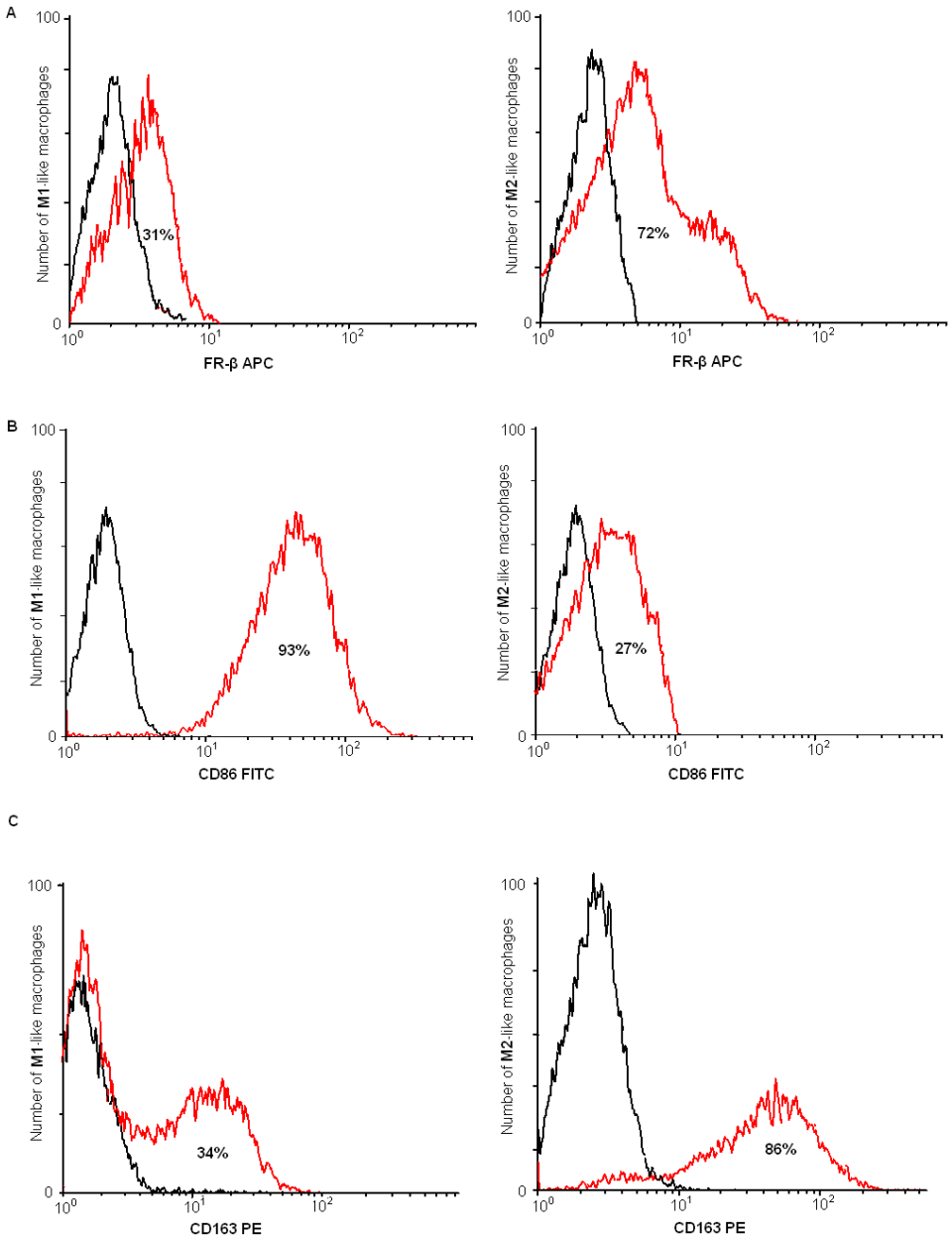


Figure 4. Representative flow cytometric analysis of M1-like and M2-like macrophage population from *in vitro* polarized macrophages, derived from PBMCs from a healthy control. (A) FR-β is expressed on 31 % of M1-like macrophages and on 72 % of M2-like macrophages (red line). (B) CD86 is expressed on 93 % of M1-like macrophages and on 27 % of M2-like macrophages (red line). (C) CD163 is expressed on 34 % on M1-like macrophages and on 86 % of M2-like macrophages (red line). Isotypes in black in all graphs.

In Vitro mRNA Expression of M1-like and M2-like Markers

mRNA levels of GAPDH, IL-6, TNF, TLR-2 and -4, FR-β, MR, IL-10 and MMP-9 were determined in M1-like and M2-like differentiated macrophages in duplicate from 6 healthy volunteers. mRNA expression of FR-β, MR, IL-10 and MMP-9 was increased in M2-like macrophages compared to M1-like macrophages (P=0.09, P=0.04, P=0.06 and P=0.004, respectively, figure 5). On the contrary, TNF and TLR-2 mRNA levels were significantly higher in M1-like macrophages compared to those in M2-like macrophages (see figure 6, P=0.002 for both). No significant difference could be found in mRNA levels of IL-6 and TLR-4 between M1-like and M2-like macrophages.

Figure 5.

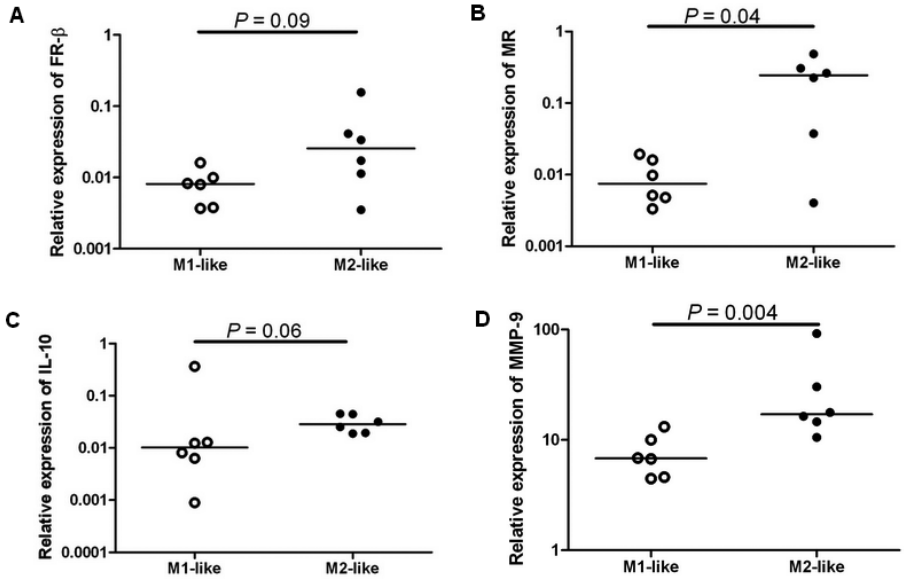


Figure 5. Relative expression of mRNA levels to GAPDH in 6 healthy volunteers. Quantitative reverse transcription polymerase chain reaction revealed a higher relative expression in M2-like macrophages (black dots) compared to M1-like macrophages (open dots) in (A) FR-β (P=0.09), (B) Mannose receptor (P=0.04), (C) IL-10 (P=0.06) and (D) MMP-9 (P=0.004).

Figure 6.

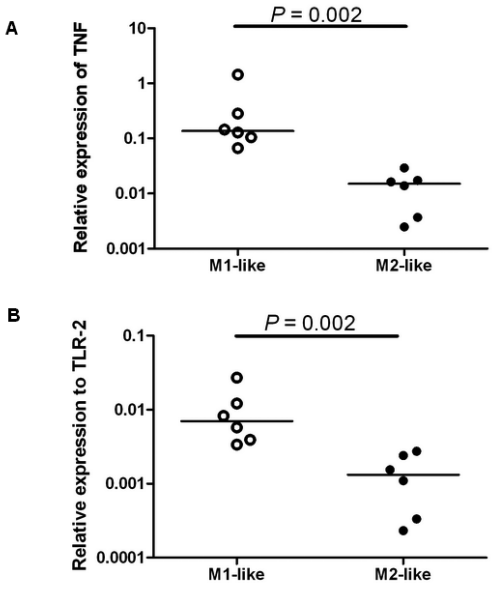


Figure 6. Relative expression of mRNA levels to GAPDH in 6 healthy volunteers measured in duplo showing a higher relative expression in M1-like macrophages (open dots) compared to M2-like macrophages (black dots) in (A) TNF (P=0.002) and (B) TLR-2 (P=0.002)

Discussion

This is the first study to show feasibility using ^{99m}Tc-folate to image human atherosclerotic carotid plaques ex vivo. Slices with the highest ^{99m}Tc-folate accumulation showed significantly more FR-β and CD163 expression compared to slices with lowest ^{99m}Tc-folate accumulation, and significantly less expression of iNOS. Therefore, FR-β positive macrophages were found to be primarily M2-like polarized macrophages, which might be pro-atherogenic, due to production of matrix degrading enzymes.

A number of research groups succeeded in specifying macrophage subsets expressing the folate receptor on tumor-associated M2 macrophages (16), and on M2 macrophages expressed in experimental allergic asthma in mice (17). Furthermore, FR-β targeted immunotoxin treatment resulted in reduction of macrophages and plaque progression (18). However, by our knowledge this is the first study to show an overexpression of FR-β on M2-like macrophages in atherosclerosis. Nevertheless, identification of macrophage subtypes and determination of their role in the atherosclerotic process is still a major challenge.

The M1 and M2 macrophage subtypes are observed in atherosclerotic lesions previously (19,20). Shaikh et al. found a higher proportion of M1 pro-inflammatory macrophages and a reduced proportion of M2-cells in symptomatic carotid lesions (21). The discrepancy

to our study could be due to the fact that the former study compared the ratio of macrophage subtypes in the carotid artery to those in vascular femoral plaques. In an experimental atherosclerotic ApoE KO mice model, lesion progression was correlated with the dominance of M1 (arginase (Arg) II^{+}) over the M2 (Arg I^{+}) macrophage subtype (22). This could be due to the proportion of M1 and M2 macrophages depending on stadium of plaque formation in the APOE model. In recent published studies other M2 markers were found to be related to worse cardiovascular outcome. In sections derived from subjects enrolled in the Cardiovascular Pathology Institute Sudden Coronary Death Registry, stable plaques revealed very few M2-like macrophages (made visible by targeting the Mannose Receptor) compared to unstable plaques where the M2-like macrophage subpopulation was abundant (23). Furthermore, patients with a 2.4 fold increased cardiovascular risk compared to patients with a low ratio, were found to have a high MMP-12/CD68 ratio (24). MMP-12 was found to be correlated with MMP-9 positive macrophage subpopulations in a mouse model for obesity (25). Stöger et al found a correlation between areas of intraplaque hemorrhage and CD163 staining in human atherosclerosis. During development of plaques both M1 and M2 cells accumulated, and the fibrous caps of lesions showed no significant differences between subsets. In contrast, vascular adventitial tissue displayed a pronounced M2 activation profile (26). These different studies support our hypothesis that accumulation of M2-like macrophages might be indicative of plaque vulnerability, which can be imaged by ^{99m}Tc-folate imaging.

Visualizing folate receptors has been performed before using optical fluorescent contrast agent (FITC) labeled to folate by our group (7). Other groups also used optical imaging for the identification of tumor processes, as several solid tumors express folate receptor α, demonstrated in ovarian cancer (27) and in head- and neck carcinoma expressing folate receptor-β (28). However, applications for non-invasive optical imaging of fluorescent signals by the use of fluorophores such as FITC could be of less clinical value because of the limited penetration depth of only a few millimeters. This could be extended to a few centimeters using near infrared (NIR) probes, but not ideal for the use in coronary arteries for instance. ^{99m}Tc-folate has a good tissue penetration and a relatively short half life. Furthermore its radiolabeling procedure is simple and it has not shown toxicity or immunogenicity (8).

For in vivo folate receptor imaging, the SPECT modality could be used; Ayala Lopez et al. tested ^{99m}Tc-folate accumulation in ApoE knockout mice on Western chow diet, and showed a significantly greater accumulation in atherosclerotic lesions than mice on normal chow (8). In humans ^{111}In-DTPA-folate and ^{99m}Tc-EC20-folate have been tested in clinical trials as radiotracer for imaging of cancer (FR-α) (29,30). Nevertheless, Winkel et al. showed ^{111}In-EC0800 (a radiolabelled folate compound) detects plaque but was not able to differentiate between a stable and a vulnerable atherosclerotic carotid plaque in a shear stress induced ApoE knockout mouse model (31). However, in this model macrophage content is not different between the oscillatory shear stress region (stable plaque) and the lowered shear

stress region (vulnerable plaque) (32). Also PET tracers have been developed recently, of which 3'-Aza-2'-[^{18}F]fluorofolic acid (33) has been shown to selectively target FR-β positive macrophages in atherosclerotic plaques (34). No determination of differential folate accumulation in the respective M1-like and M2-like subpopulation was performed in this study. [^{18}F]fluoro-PEG-folate has been used in a rat model of arthritis to image (sub)clinical arthritis (35). For imaging of plaque inflammation 2-deoxy-2-[^{18}F]fluoro-D-mannose ([^{18}F] FDM), has been developed recently, supporting a higher accumulation in macrophages compared to [^{18}F]FDG (23).

A limitation of our study is that division of macrophages solely as M1 or M2 subtypes may be too simple. As Wolfs et al. suggests the role of the local micro-environment makes macrophage polarization in the atherosclerotic tissue more complex than the typically described M1 and M2 macrophages distribution (11). So, not only cytokine environment, but also foam cell formation and CXCL4 (chemokine ligand 4, forming M4 macrophages), among other factors, play major roles. This could be an explanation for the fact that in Figure 2A a CD68+ population of macrophages in the plaque does not stain for the M1/M2 markers or FR-β. Further studies to investigate the release of matrix metalloproteinases by macrophage subtypes may give more insight. Also, due to the inclusion of a low number of asymptomatic patients, no analyses between the ratio of M1-like and M2-like macrophages in symptomatic and asymptomatic disease could be made. More research is necessary to explore this issue.

In conclusion, this is the first study showing a positive correlation between accumulation of a ^{99m}Tc-folate compound in atherosclerotic plaques and FR-β expression on M2-like macrophages. Furthermore we characterized macrophage phenotypes *ex vivo* and *in vitro* by a panel of macrophage subtype markers. This not only enables the specific analysis of the proportion of macrophage subtypes within a plaque, but also opens ways to measure M2-like macrophage populations *ex vivo*. More insight in macrophage subtypes and behavior in atherosclerotic plaques may give a better understanding of the pathogenesis related to the vulnerability of atherosclerotic plaques.

Acknowledgements

We thank Fleur Schaper, Gerda Horst and Gerda de Vries for measuring macrophage markers using flow cytometry, Irene Zwarts for measuring macrophage markers using RT-PCR, and Berber Doornbos for performing immunohistochemical stainings.

References

(1) World Health Organization. The top 10 causes of death. Fact sheet no. 31; http://www.who.int/mediacentre/factsheets/fs310/en/index.html. 2011; .
(2) Finn AV, Nakano M, Narula J, Kolodgie FD, Virmani R. Concept of vulnerable/unstable plaque. Arterioscler Thromb Vasc Biol 2010 Jul;30(7):1282-1292.
(3) Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011 May 19;473(7347):317-325.
(4) Zeebregts CJ, Meerwaldt R, Geelkerken RH. Carotid artery stenting: a 2009 update. Curr Opin Cardiol 2009 Nov;24(6):528-531.
(5) Hermus L, van Dam GM, Zeebregts CJ. Advanced carotid plaque imaging. Eur J Vasc Endovasc Surg 2010 Feb;39(2):125-133.
(6) Masteling MG, Zeebregts CJ, Tio RA, Breek JC, Tietge UJ, de Boer JF, et al. High-resolution imaging of human atherosclerotic carotid plaques with micro 18F-FDG PET scanning exploring plaque vulnerability. J Nucl Cardiol 2011 Dec;18(6):1066-1075.
(7) Jager NA, Westra J, van Dam GM, Teteloshvili N, Tio RA, Breek JC, et al. Targeted folate receptor beta fluorescence imaging as a measure of inflammation to estimate vulnerability within human atherosclerotic carotid plaque. J Nucl Med 2012 Aug;53(8):1222-1229.
(8) Ayala-Lopez W, Xia W, Varghese B, Low PS. Imaging of atherosclerosis in apoliprotein e knockout mice: targeting of a folate-conjugated radiopharmaceutical to activated macrophages. J Nucl Med 2010 May;51(5):768-774.
(9) Medbury HJ, James V, Ngo J, Hitos K, Wang Y, Harris DC, et al. Differing association of macrophage subsets with atherosclerotic plaque stability. Int Angiol 2013 Feb;32(1):74-84.
(10) Jager NA, Teteloshvili N, Zeebregts CJ, Westra J, Bijl M. Macrophage folate receptor beta (FR-beta) expression in auto-immune inflammatory rheumatic diseases: A forthcoming marker for cardiovascular risk? Autoimmun Rev 2011 Nov 7.
(11) Wolfs IM, Donners MM, de Winther MP. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost 2011 Nov;106(5):763-771.
(12) Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004 Dec;25(12):677-686.
(13) Meerwaldt R, Hermus L, Reijnen MM, Zeebregts CJ. Carotid endarterectomy: current consensus and controversies. Surg Technol Int 2010 Oct;20:283-291.
(14) Loening AM, Gambhir SS. AMIDE: a free software tool for multimodality medical image analysis. Mol Imaging 2003 Jul;2(3):131-137.
(15) Boyle JJ, Harrington HA, Piper E, Elderfield K, Stark J, Landis RC, et al. Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. Am J Pathol 2009 Mar;174(3):1097-1108.
(16) Puig-Kroger A, Sierra-Filardi E, Dominguez-Soto A, Samaniego R, Corcuera MT, Gomez-Aguado F, et al. Folate receptor beta is expressed by tumor-associated macrophages and constitutes a marker for M2 anti-inflammatory/regulatory macrophages. Cancer Res 2009 Dec 15;69(24):9395-9403.
(17) Shen J, Chelvam V, Cresswell G, Low PS. Use of folate-conjugated imaging agents to target alternatively activated macrophages in a murine model of asthma. Mol Pharm 2013 May 6;10(5):1918-1927.
(18) Furusho Y, Miyata M, Matsuyama T, Nagai T, Li H, Akasaki Y, et al. Novel Therapy for Atherosclerosis Using Recombinant Immunotoxin Against Folate Receptor beta-Expressing Macrophages. J Am Heart Assoc 2012 Aug;1(4):e003079.
(19) Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S, et al. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell

Metab 2007 Aug;6(2):137-143.
(20) Finn AV, Nakano M, Polavarapu R, Karmali V, Saeed O, Zhao X, et al. Hemoglobin directs macrophage differentiation and prevents foam cell formation in human atherosclerotic plaques. J Am Coll Cardiol 2012 Jan 10;59(2):166-177.
(21) Shaikh S, Brittenden J, Lahiri R, Brown PA, Thies F, Wilson HM. Macrophage subtypes in symptomatic carotid artery and femoral artery plaques. Eur J Vasc Endovasc Surg 2012 Nov;44(5):491-497.
(22) Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, Clement M, et al. Macrophage plasticity in experimental atherosclerosis. PLoS One 2010 Jan 25;5(1):e8852.
(23) Tahara N, Mukherjee J, de Haas HJ, Petrov AD, Tawakol A, Haider N, et al. 2-deoxy-2-[(18)F]fluoro-d-mannose positron emission tomography imaging in atherosclerosis. Nat Med 2014 Feb;20(2):215-219.
(24) Scholtes VP, Johnson JL, Jenkins N, Sala-Newby GB, de Vries JP, de Borst GJ, et al. Carotid atherosclerotic plaque matrix metalloproteinase-12-positive macrophage subpopulation predicts adverse outcome after endarterectomy. J Am Heart Assoc 2012 Dec;1(6):e001040.
(25) Shaul ME, Bennett G, Strissel KJ, Greenberg AS, Obin MS. Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet--induced obesity in mice. Diabetes 2010 May;59(5):1171-1181.
(26) Stoger JL, Gijbels MJ, van der Velden S, Manca M, van der Loos CM, Biessen EA, et al. Distribution of macrophage polarization markers in human atherosclerosis. Atherosclerosis 2012 Dec;225(2):461-468.
(27) van Dam GM, Themelis G, Crane LM, Harlaar NJ, Pleijhuis RG, Kelder W, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-alpha targeting: first in-human results. Nat Med 2011 Sep 18.
(28) Sun JY, Shen J, Thibodeaux J, Huang G, Wang Y, Gao J, et al. In Vivo optical imaging of folate receptor-beta in Head and Neck squamous cell carcinoma. Laryngoscope 2014 Jan 21.
(29) Fisher RE, Siegel BA, Edell SL, Oyesiku NM, Morgenstern DE, Messmann RA, et al. Exploratory study of 99mTc-EC20 imaging for identifying patients with folate receptor-positive solid tumors. J Nucl Med 2008 Jun;49(6):899-906.
(30) Edelman MJ, Harb WA, Pal SE, Boccia RV, Kraut MJ, Bonomi P, et al. Multicenter trial of EC145 in advanced, folate-receptor positive adenocarcinoma of the lung. J Thorac Oncol 2012 Oct;7(10):1618-1621.
(31) Winkel LC, Groen HC, van Thiel BS, Muller C, van der Steen AF, Wentzel JJ, et al. Folate Receptor-targeted Single-Photon Emission Computed Tomography/Computed Tomography to Detect Activated Macrophages in Atherosclerosis: Can It Distinguish Vulnerable from Stable Atherosclerotic Plaques? Mol Imaging 2013;12(0):1-5.
(32) Cheng C, Tempel D, van Haperen R, van der Baan A, Grosveld F, Daemen MJ, et al. Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. Circulation 2006 Jun 13;113(23):2744-2753.
(33) Betzel T, Muller C, Groehn V, Muller A, Reber J, Fischer CR, et al. Radiosynthesis and preclinical evaluation of 3′-Aza-2′-[(18)F]fluorofolic acid: a novel PET radiotracer for folate receptor targeting. Bioconjug Chem 2013 Feb 20;24(2):205-214.
(34) Muller A, Beck K, Rancic Z, Muller C, Fischer CR, Betzel T, et al. Imaging Atherosclerotic Plaque Inflammation via Folate Receptor Targeting Using a Novel 18F-Folate Radiotracer. Mol Imaging 2014 Jan 1;13(1):1-11.
(35) Gent YY, Weijers K, Molthoff CF, Windhorst AD, Huisman MC, Smith DE, et al. Evaluation of the novel folate receptor ligand [18F]fluoro-PEG-folate for macrophage targeting in a rat model of arthritis. Arthritis Res Ther 2013 Mar 1;15(2):R37.

CHAPTER 8

Distribution of matrix metalloproteinase's in human atherosclerotic carotid plaques and the role of smooth muscle cells and macrophage polarization in their production.

Nynke A. Jager, Bastiaan M. Wallis de Vries, Jan-Luuk Hillebrands, Niels J. Harlaar, René A. Tio, Riemer H.J.A. Slart, Gooitzen M. van Dam, Hendrikus H. Boersma, Clark J. Zeebregts, Johanna Westra

Submitted for publication

Abstract

OBJECTIVES Matrix metalloproteinases (MMPs) destabilize atherosclerotic plaques by promoting matrix destruction, angiogenesis and leukocyte infiltration. Macrophages and smooth muscle cells (SMCs) are involved in the development of vulnerable plaques, as they produce MMPs. AIM In this study we explored the potential of MMPsense as marker for atherosclerotic plaque instability using molecular imaging techniques. Secondly, presence of different MMPs in atherosclerotic plaques, and expression of MMPs by macrophage subtypes and SMCs was investigated.

METHODS Twenty-three consecutive plaques removed during carotid endarterectomy were incubated in a MMP-sensitive activatable fluorescent probe (MMPSense™ 680) and multispectral fluorescence imaging with IVIS® Spectrum was performed. mRNA levels of MMPs, macrophage markers, and SMCs were determined in specimens of plaques. mRNA expression of different MMPs was determined in *in vitro* differentiated M1 and M2 macrophages from healthy volunteers and in SMCs. Furthermore, MMP-9 protein was measured in supernatants of cultured macrophages with ELISA.

RESULTS There was a significant difference between autofluorescence signals and MMPSense signals, both on the intra- and extraluminal sides of plaques. MMP-9 and CD68 mRNA expression was higher in hot spots than in cold spots, whereas MMP-2 and αSMA expression was higher in cold spots. In plaques, MMP-9 mRNA expression was 100-1000 fold higher than other MMPs and correlated strongly with CD68 expression. *In vitro* M2 macrophages had higher mRNA expression of MMP-1, -9, -12 and TIMP-1 and lower MMP-2 and MMP-14 expression compared to M1 macrophages.

CONCLUSION MMP-9 is most dominantly MMP present in atherosclerotic plaques and is produced by M2 rather than M1 macrophages. MMPsense can be used to detect MMP-9 in plaques and might be a good marker to investigate plaque instability in the clinical setting.

Introduction

Atherosclerosis is a progressive inflammatory disease characterized by the accumulation of lipid-filled macrophages within the arterial intima. Continued inflammation may promote rupture of the atherosclerotic plaque's protective fibrous cap causing subsequent clinical ischaemic events (1,2). The fibrous cap covering an advanced atherosclerotic plaque is typically composed of smooth muscle cells (SMC) and extracellular matrix (ECM) (3). Activated monocyte-derived macrophages and SMCs are critically involved in the development of high-risk vulnerable plaques by producing matrix metalloproteinases (MMP's) (1,4,5).

A heterogeneous population of macrophages exists including a classically activated macrophage type (M1) as well as an alternatively activated macrophage population (M2) (6). The M1 macrophage is thought to have pro-inflammatory properties and polarization *in vitro* is driven by low concentration lipopolysacharride (LPS) and interferon gamma (IFN-γ). Defined as classically however, M2 macrophages are anti-inflammatory and immune regulatory. Upon cytokine stimulation they modify development of atherosclerotic plaques. The M2 macrophage population can be divided in M2a, M2b and M2c subtypes, depending on the cytokine environment (IL-4, immune complexes and IL-10, respectively) (7). In contrast to its classical M2-properties, the function of M2a macrophages is wound-healing or tissue-repair functions, and the function of type M2c is matrix deposition and tissue remodeling; this last mentioned type might be most important in atherosclerosis (8). Recently, Wolfs et al. suggested that additional circumstancesin the local micro-environment makes macrophage polarization in the atherosclerotic tissue even more complex than the typically described M1 and M2 macrophages distribution (9). So, not only cytokine environment, but also foam cell formation and CXCL4 (chemokine ligand 4), among other factors, play major roles.

MMP's are proteolytic enzymes that can degrade ECM proteins such as gelatin (MMP-2 and -9), collagen (MMP-1, -8 and -13), elastin (MMP-12) and fibrin (MMP-3 and -10). They can be inhibited by tissue inhibitors of metalloproteinases (TIMP's). The ever growing MMP family now consists of more than 20 known proteins (10). There is conflicting evidence about the role of MMP-9 in atherosclerosis. In carotid atherosclerotic disease MMP-9 is mostly associated with the development of unstable plaques. In patients with symptomatic carotid disease, increased MMP-9 levels have been shown both in plasma and in plaque tissue (11-16). Other studies showed an inverse relation between plaque activity and MMP-9 plasma levels, or even evidence for a plaque stabilizing role for MMP-9 (17,18). At the moment, MMP's cannot be visualized with conventional imaging modalities, such as duplex ultrasound, computerized tomography scanning, and magnetic resonance imaging. Although these conventional imaging techniques have improved and do have the ability to image cardiovascular anatomy and physiology on a macroscopic scale, they lack the possibility to detect biological processes at the cellular or molecular level (19). In contrast,

molecular imaging has the possibility to target molecular components of atherosclerotic disease on a microscopic scale using smart activatable probes (20). As such, MMPs may be targeted with a MMP-sensitive probe (MMPsense) and can be visualized by fluorescence imaging (21-23). MMPsense is a protease activatable fluorescent imaging agent that is activated by MMP -2, -3, -9 and -13. MMPsense is optically silent in its inactivated state and becomes highly fluorescent following protease-mediated activation.

In this study, we analyzed the presence of MMPs in human carotid plaques by using fluorescence and investigated the differences in intensity of fluorescence signals. Furthermore, macrophages were differentiated and polarized *in vitro* into M1, M2a and M2c macrophages to investigate expression of MMPs in these subtypes, as well as in smooth muscle cells. In this way the potential of MMPsense as marker for atherosclerotic carotid plaque vulnerability was explored, and the relation to MMP expression of macrophage subtypes and smooth muscle cells throughout atherosclerotic plaques.

Materials and Methods

Study Design

Patients presenting with symptoms (i.e. with a history of recent cerebrovascular accident (CVA)), transient ischaemic attack (TIA) or amaurosis fugax) and a stenosis of the common carotid artery of 70-99% as detected by duplex ultrasound examination underwent open carotid surgery at the University Medical Center Groningen (UMCG). Additionally, asymptomatic patients with a stenosis of 80-99%, found by routine control were also eligible for surgical treatment. Therefore, a total of 23 carotid specimens were obtained by means of carotid endarterectomy (CEA) of the carotid bifurcation using standard techniques (24). Risk factors such as hyperlipidemia, hypertension, smoking status, obesity (body mass index, BMI) and diabetes mellitus were recorded. Hyperlipidemia and hypertension was defined as described before by our group (25). To measure MMP expression in differentiated macrophages, ten healthy volunteers were included without known cardiovascular disease or risk factors. The study was approved by the Institutional Review Board (IRB) of the UMCG and informed consent was obtained from all patients and healthy volunteers.

Carotid Endarterectomy Sample Collection and Timepath of Study

Plaques were obtained immediately after endarterectomy. Following endarterectomy all specimen were washed with PBS to remove blood and debris. After storage in PBS, samples were put on ice and taken to the laboratory for fluorescence imaging. The plaques were cut open longitudinally and pinched on a silicone plate with non reflective black paper (XPB-24 black paper, Caliper Life Sciences, Hopkinton, MA, USA) in between to reduce autofluorescence. Then, autofluorescence signals were recorded on the intra- and extraluminal sides of the plaque. The mean time between excision of the plaque and

determination of autofluorescence was 31 minutes (16-45). It took 9 minutes (2-26) to complete the autofluorescence recordings. After that, the plaque was incubated with a MMP-sensitive activatable fluorescent probe (MMPSense™680, VisEn Medical, Boston, MA, USA), which was diluted 1:10. Specimens were incubated for 66 minutes (60-72) before imaging.

Fluorescence imaging and data analysis

Fluorescence images were obtained with a commercial imaging system with an ultra-sensitive charge-coupled device camera mounted on a light-tight black chamber (IVIS® spectrum, Caliper Life Sciences, Hopkinton, MA, USA). The charged coupled device (CCD) camera was cooled to -90^0 Celcius. The excitation and emission filter were set at Cy5.5. By dividing the mean radiance (p/s/cm^2/sr) from the MMPSense signal by the autofluorescence signal the target-to-background ratio (TBR) was calculated. The imaging data were analyzed using Living Image 3.0 software (Caliper Life Sciences, Hopkinton, MA, USA).

Immunohistochemistry

Slices of plaques were embedded in paraffin, and sections of 4 µm were cut. Macrophages were identified by incubation with monoclonal mouse anti-human CD68 (1:50; mo876 clone PG-M1 DAKO, Glostrup, Denmark). For detection of MMP-9, a goat anti-human antibody (AB911, R&D systems, Minneapolis, USA) was used. Appropriate secondary antibodies labeled with horseradish peroxidase (HRP) were used. Color reaction was developed using DAB staining with chromogen, and sections were counterstained with hematoxylin.

***In Vitro* Differentiation and Polarization of M1, M2a and M2c Macrophages**

From ten healthy donors peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Axis Shield PoC As, Oslo, Norway). Subsequently, monocytes were allowed to adhere to culture plates. The adherent cells were maintained for 5 days in RPMI 1640 medium (Lonza, Walkersville, MD, USA) supplemented with 10% filtered fetal calf serum (FCS) and 20 ng/ml macrophage colony stimulating factor (M-CSF, R&D systems) for differentiation into macrophages. Macrophages were directed towards M1, M2a and M2c phenotypes by use of 48 hours stimulation with 100 U/ml IFNγ (PeproTech, USA) and 1 ng/ml LPS (Sigma, Germany), 20 ng/ml IL-4 and/or 10ng/ml IL-10 (PeproTech), respectively. MMP-9 protein was measured in supernatants of cultured macrophages with ELISA (duoset, R&Dsystems) according to manufacturer's description.

RNA expression *in vitro* in macrophage subtypes and SMCs and *ex vivo* in plaques

To measure MMP expression, RNA was extracted from above named macrophage subtypes and from smooth muscle cells as described before (25). From four imaged plaques areas

with high intensity (hotspots) and low intensity (cold spots) were excised and mRNA was isolated from these specimens. Four un-imaged plaques were divided in equal slices of 5 mm, and mRNA was also isolated from these slices. cDNA samples were added in duplicate for amplification by the Taqman real time PCR system (ABI Prism 7900HT Sequence Detection system, Applied Biosystems, Foster City, CA, USA). mRNA expression of MMP-1, -2, -3, -8, -9, -12, -13, -14 and -16, and TIMP-1 was measured by using Taqman primer/probes sets. In slices of plaques, also mRNA expression of CD68 (pan macrophages), CD86 (M1 macrophage marker), CD163 (M2 macrophage marker) and αSMA (ACTA2) was investigated. Ct (threshold cycle) values were determined using the software program SDS 2.4 (Applied Biosystems). Relative gene expression was normalized to the expression of GAPDH and calculated by the following formula: relative gene expression = $2^{-\Delta Ct}$ (ΔCt = Ct gene of interest – Ct GAPDH).

Statistical Analysis

Values are presented as mean ± standard deviation or median (range), unless stated otherwise. For correlations, Pearson's and Spearman's correlation coefficients were used when appropriate. A two-tailed, paired student T-test was used for parametric distributions (i.e. fluorescence signal and TBR)). Non-paired continuous variables with a non-parametric distribution were analyzed using the Mann-Whitney U-test. For comparing more than two independent samples, the Kruskal-Wallis test was used (i.e. four types of macrophages). A two-sided P value < 0.05 was considered statistically significant. Statistical tests were done with the Statistical Package for the Social Sciences (SPSS statistics version 20.0, SPSS inc®, Chicago IL, USA).

Results

Patient Demographics

A total of 15 men and eight women with a mean age of 70 ± 9 years were included. Baseline characteristics of participants are shown in Table 1.

Table 1. Baseline characteristics and risk factors for atherosclerosis

	Patients (n=23)
Men, n (%)	15 (65)
Age, years	70 ± 9
Symptomatic, n (%)	21 (91)
Transient ischemic attack (TIA), n (%)	9 (39)
Cerebro vascular accident (CVA), n (%)	8 (35)
Amaurosis fugax, n (%)	4 (17)
BMI, kg/m2	27 ± 3
Smoking status, n (%)	10 (43)
> 1 pack a day, n (%)	6 (26)
≤ 1 pack a day, n (%)	4 (17)
None, smoked in last 10 years (%)	6 (26)
Hypertension, n (%)	19 (83)
Controlled with single drug, n (%)	5 (21)
Controlled with 2 drugs, n (%)	5 (21)
Requires > 2 drugs or uncontrolled, n (%)	9 (39)
Systolic blood pressure, mmHg	146 ± 22
Diastolic blood pressure, mmHg	80 ± 12
Hyperlipidemia, n (%)	14 (61)
Use of lipid-lowering drugs, n (%)	9 (39)
Diabetes mellitus, n (%)	7 (30)*

* Four patients had diabetes controlled by diet or oral agents, three patients where on insulin. Data are expressed as mean ± standard deviation; percentages between brackets.

Fluorescence imaging

Fluorescence signal of each plaque was recorded before and after incubation with MMPSense™680. MMP signals were heterogeneously distributed throughout plaques (Figure 1). The mean TBR was considered appropriate, and did not significantly differ between intraluminal and extraluminal sides (7.15 vs 6.43; P=0.53). Fluorescence signals clearly augmented after incubation with MMPSense compared to autofluorescence, showing highly significant differences on both intraluminal (6.34 vs 1.09; P<0.0001) and extraluminal sides (6.12 vs 1.04; P<0.0001) (Figure 2).

Figure 1.

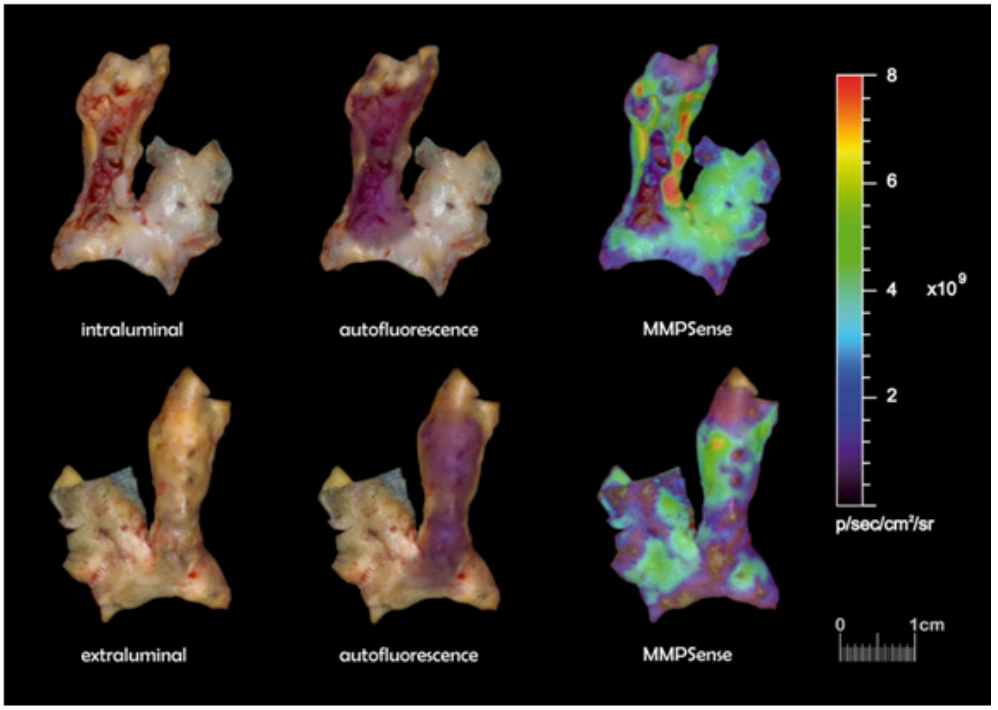


Figure 1. Sections of fluorescence image of an atherosclerotic carotid plaque specimen after MMPSense incubation (as well as autofluorescence) showing heterogeneous accumulation on intra- and extra luminal side.

Figure 2.

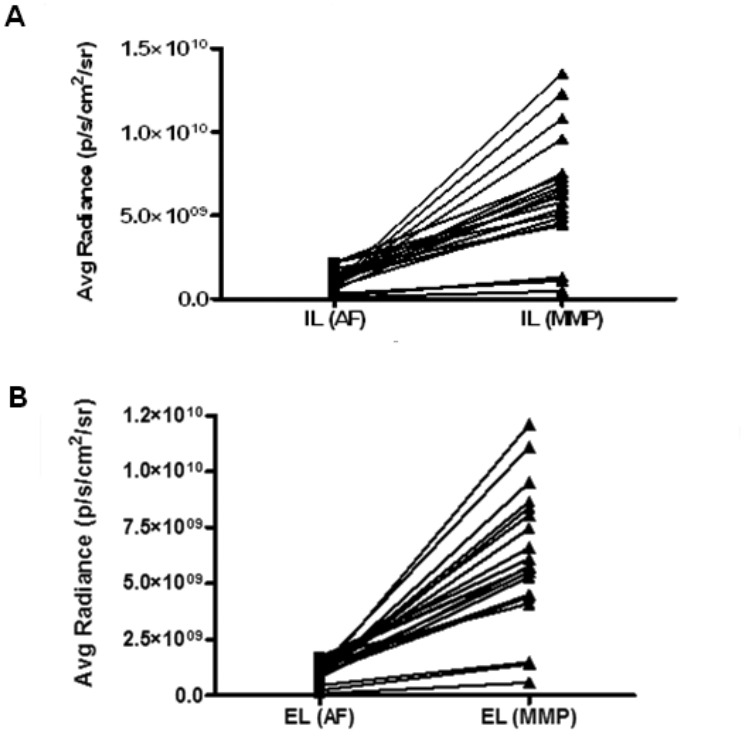


Figure 2. Fluorescence signals after incubation with MMPSense compared to autofluorescence. (A) intraluminal (6.34 vs 1.09; p<0.0001) and (B) extraluminal side (6.12 vs 1.04; p<0.0001).

***Ex vivo* MMP, αSMA and macrophage expression in plaques**

MMP-2, MMP-9, αSMA and CD68 mRNA expression was investigated in hot and cold spots from 4 imaged plaques. As can be seen in figure 3A, MMP-9 and CD68 expression was up regulated in hot spots, whereas MMP-2 and αSMA were down regulated in hot spots. To investigate the expression and distribution of MMPs, and their relation to M1 and M2 macrophages in plaques, mRNA expression of MMPs was compared to CD68 (pan macrophages), and to an M1 marker (CD86) and an M2 macrophage marker (CD163). Also, MMP expression was compared to αSMA expression. The strongest correlation was found between MMP-9 and CD68 mRNA expression (P< 0.001, Figure 3B). Furthermore, MMP-9 expression was 100 to 1000 times higher compared to mRNA expression of other MMPs (data not shown) None of the other MMPs showed a significant correlation with macrophage markers, except for MMP-2 and MMP-14 which both correlated significantly with CD86 and CD163. There was a significant correlation between MMP-2 and αSMA expression (Figure 3B). Immunohistochemical staining of plaques for CD68 and MMP-9 showed overlap of CD68 and MMP-9 as can be seen in a representative picture in figure 3C.

Figure 3.

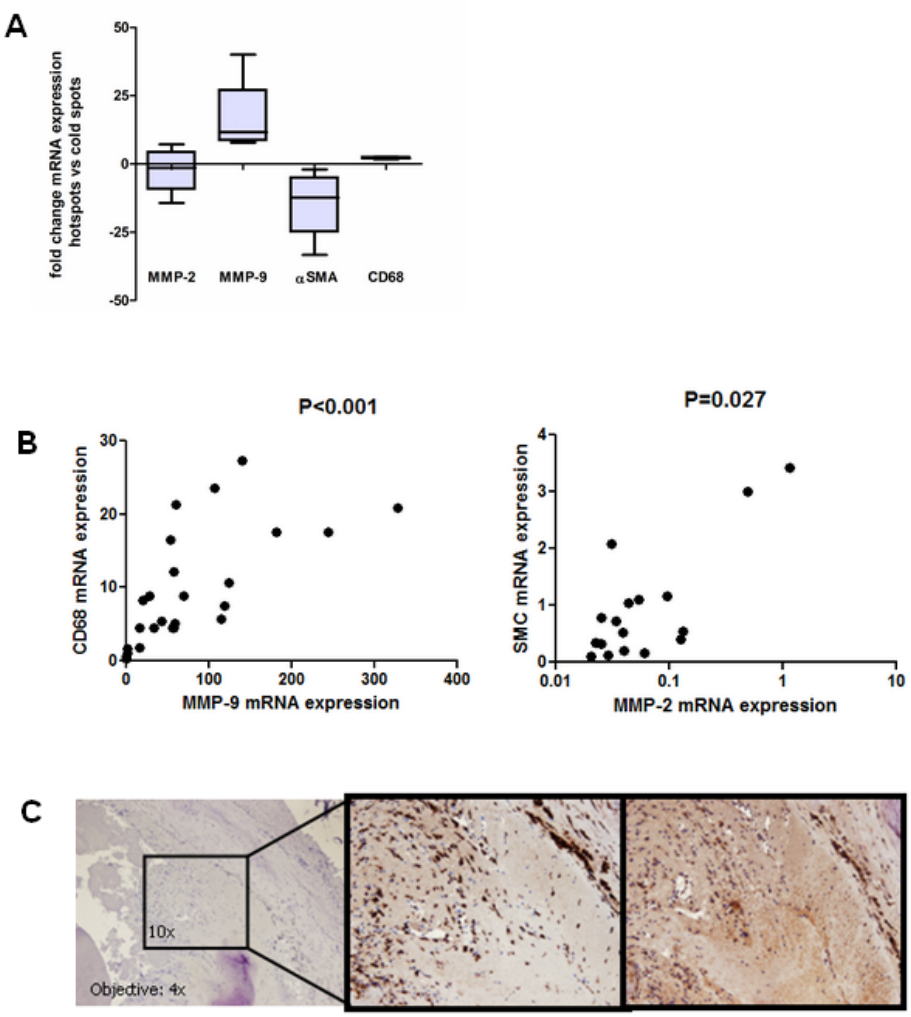


Figure 3. Expression of MMPs in atherosclerotic plaques. (A) Fold change mRNA expression in hot spots versus cold spots. (B) Hot spots and cold spots from 4 imaged plaques were excised and mRNA expression of MMP-2, MMP-9, αSMA and CD68 was determined with RT-PCR. Correlation between MMP-9 and CD68 mRNA expression and between MMP-2 and αSMA mRNA expression in slices of plaques. (C) Co localization of immunohistochemical staining of CD68 and MMP-9 in plaques.

8

MMP expression in macrophage subtypes and SMCs *in vitro*

mRNA levels of GAPDH (household gene), MMP-1, -2, -3, -8, -9, -12, -13, -14 and -16, and TIMP-1 were determined in M1, M2a, M2c and IL-4 / IL-10 differentiated M2 macrophages from 10 healthy volunteers. mRNA expression of MMP-1 was significantly increased in all three M2 macrophage types compared to M1 macrophages (P<0.05, Paired T-test for all,

Figure 4A). TIMP-1 expression was significantly decreased in M1 macrophages compared to M2 macrophages (P<0.001, Figure 4B). MMP-9 and MMP-12 mRNA was undetectable in SMCs. MMP-9 expression was higher in M2 macrophages compared to M1 (Figure 4C). Only the difference between M1 and M2c macrophages was statistically significant (Paired T-test, P=0.02). MMP-12 was significantly higher expressed in M2 compared to M1 macrophages (P<0.001, Figure 4D). On the contrary, MMP-2 and MMP-14 were significantly higher in M1 macrophages compared to all types of M2 macrophages (P<0.02, Figure 5). Of note, MMP-1 and MMP-2 expression was high in SMCs. MMP-3, -8, -13 and -16 were undetectable in *in vitro* generated macrophages and in SMCs. From mRNA data it was shown that expression of MMP-9 was 100 to 1000 times higher compared to mRNA expression of other MMPs (figure 4C). Therefore MMP-9 protein secretion was investigated in supernatants of cultured macrophages. As can be seen in figure 6, all different types of macrophages can produce MMP-9 although M2 macrophages produce more than M1 (not significantly different). So protein levels of MMP-9 are comparable to the mRNA expression of MMP-9 in different subtypes of macrophages.

Figure 4.

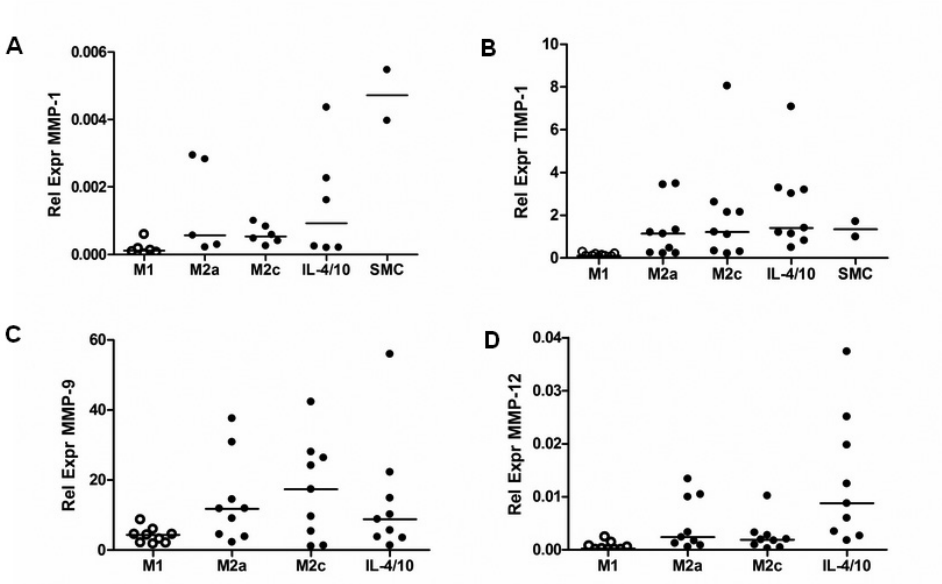


Figure 4. Relative expression of MMP mRNA levels in macrophages from 10 healthy volunteers and in SMCs.

Significantly higher relative expression in M2-like macrophages (black dots) compared to M1-like macrophages (open dots) was revealed by quantitative reverse transcription polymerase chain reaction in (A) MMP-1, (B) TIMP-1 and (D) MMP-12. MMP-9 was also higher (C).

Figure 5.

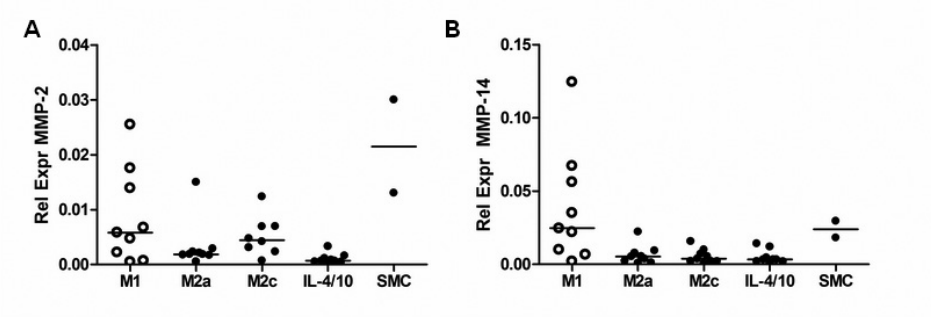


Figure 5. Relative expression of MMP mRNA levels in macrophages from 10 healthy volunteers and in SMCs. Significantly lower relative expression in M2 macrophages (black dots) compared to M1-like macrophages (open dots) was revealed by quantitative reverse transcription polymerase chain reaction in (A) MMP-2, and (B) MMP-14

Figure 6.

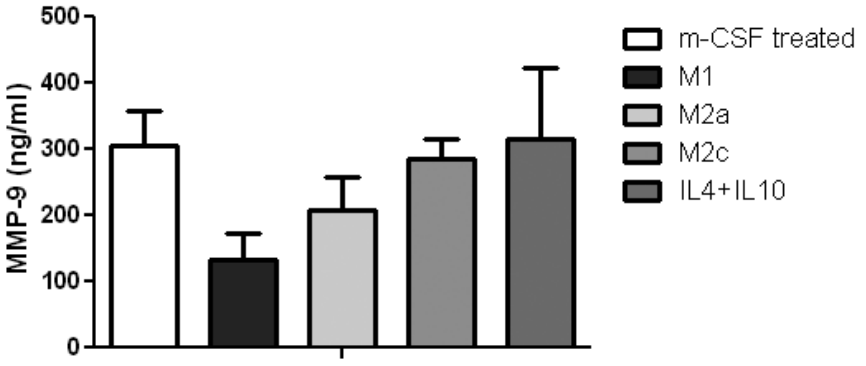


Figure 6. MMP-9 protein levels in supernatants of cultured M1 and M2 macrophages measured by ELISA.

Discussion

Our study shows that fluorescence imaging with a smart MMP-sensitive activatable probe clearly reveals a heterogeneous distribution of MMPs across the atherosclerotic carotid plaques. The signals of ex vivo human carotid plaques were significantly enhanced after incubation with the fluorescent probe accounting for a 6- to 7 fold increase of signals. MMP-9 was found to be highest expressed in plaques and in different subtypes of macrophages. Signal enhancements such as with MMPsense have been described using other protease probes, both in ex vivo carotid specimens (22), and in vivo rabbits (26). Typically, there are more intense signals (hot spots) near the carotid bifurcation. In a previous study, segments at or near the bifurcation and segments with intraplaque hemorrhage contained higher MMP levels and activity (especially MMP-9) compared to segments distant from the bifurcation. Histologically stable plaques contained lesser amounts of MMPs, which were predominantly MMP-2. TIMPs were highly abundant in fibrotic and necrotic segments (27). In a previous study by Wallis-de Vries using MMPsense in atherosclerotic plaques, and also in the present study it was shown that in areas with high intensity (hot spots) mRNA expression of MMP-9 was increased compared to areas with low intensity (cold spots) and accompanied with a slight increase in CD68 mRNA expression (23). mRNA expression of MMP-2 was decreased in hot spots compared to cold spots, and also αSMA expression. Also good correlations in plaques between mRNA expression of MMP-9 and CD68, and between MMP-2 and SMCs were found. These data are supported by another study, where macrophage rich lesions showed higher MMP-9 activity while SMC-rich lesions showed higher MMP-2 activity (28). Also, SMCs were found in stable plaques in other studies (28,29). MMP-9 protein is abundantly produced by macrophages and is abundantly present in plaques as shown by immunohistochemical staining. In a study by Loftus *et al.* the character, level, and expression of MMPs in carotid plaques was correlated to clinical status of patients undergoing carotid endarterectomy. The MMP-9 concentration was significantly higher in patients developing symptoms within one month compared to asymptomatic patients (11). Also in a study done by Heo *et al.* plaque rupture was significantly associated with the development of vascular events in carotid atherosclerotic disease, and with immunohistochemical expression of MMP-2 and -9 (30). One explanation for this might be that MMP-2 and -9 are capable of degrading collagen type IV which is the major component of the basement membrane (14). No difference in the levels of MMP-1, -2, or -3 was found between symptomatic and asymptomatic patients (11). It was also anticipated that serum levels of MMP-9 and MMP-2 were significantly higher in symptomatic patients compared to patients without symptoms (12,13). However, another group found serum MMPs where not predictive of local events, in a group of eighteen patients (31). In the *in vitro* study mRNA expression of MMP-2 was highest in pro-inflammatory M1 macrophages and in SMCs. MMP-9 and MMP-12 mRNA was highest in M2 macrophages and could not be found in SMCs. This was supported by other groups who found an over expression of MMP-9 in M2 macrophages (32), and

differentiation towards M2 macrophages up regulated MMP-12 expression (4). However, Newby *et al.* also suggest that each macrophage subtype (not only M2) can be acted on by pro-inflammatory mediators leading to activated states (4). Further research is needed to fully understand the mechanism of MMPs produced by macrophages in the process of an atherosclerotic plaque becoming vulnerable. Applications for non-invasive optical imaging of fluorescent signals could be of less clinical value in coronary atherosclerosis because of the limited penetration depth of only a few millimeters. Therefore we started testing a radiolabeled MMP tracer in *ex vivo* atherosclerotic plaques recently, from which the results look promising.

We found MMP-9 mRNA expression in plaque tissue was 100 to 1000 times higher compared to other MMPs. These findings strongly suggest that in hot spots MMPsense most likely is activated by MMP-9. Taken everything into account it seems that MMPsense can be used to detect areas of plaque instability primarily by detection of MMP-9 produced by M2 macrophages.

Conclusion

It is feasible to image MMP-9 in atherosclerotic tissue *ex vivo* using a smart activatable fluorescence probe and fluorescence imaging. MMP-9 is produced by macrophages and is abundantly present in plaques as shown by immunohistochemical staining and qRT-PCR. Furthermore, areas with high intensity (hot spots) have a higher mRNA expression of MMP-9 compared to areas with low intensity (cold spots). Also, MMP-9 expression was highest in M2 macrophages and could not be found in SMCs. In conclusion, MMPsense can be used to detect MMP-9 in plaques and might therefore be a good marker to investigate plaque instability.

Acknowledgements

We thank Ming K. Lam for assistance during plaque imaging procedures, Berber Doornbos for measuring MMPs and macrophage markers using RT-PCR in macrophages *in vitro*, and Sebastiaan ter Horst for performing immunohistochemical stainings and measuring MMPs and macrophage markers using RT-PCR in plaques.

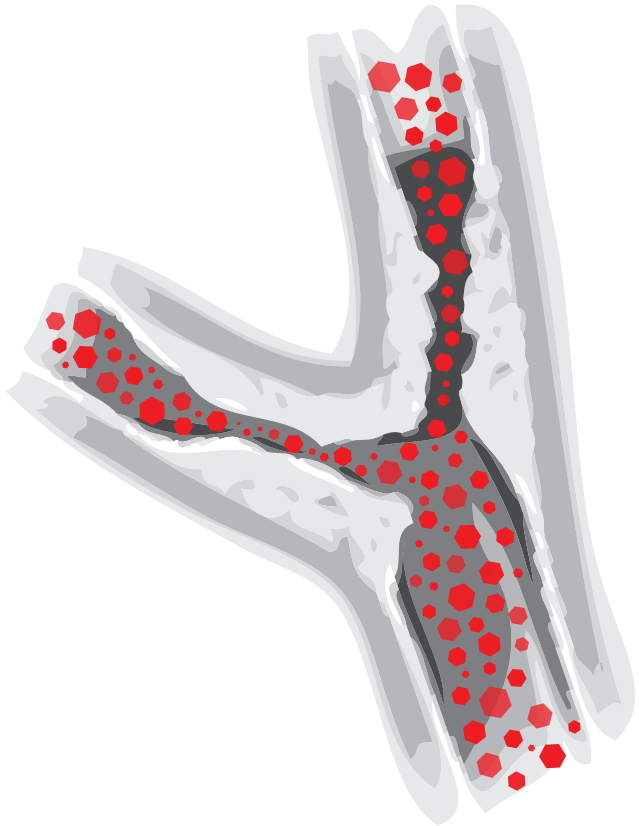
References

(1) Libby P. Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol 2012 Sep;32(9):2045-2051.
(2) Shah PK. Inflammation and plaque vulnerability. Cardiovasc Drugs Ther 2009 Feb;23(1):31-40.
(3) Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999 Jan 14;340(2):115-126.
(4) Newby AC, George SJ, Ismail Y, Johnson JL, Sala-Newby GB, Thomas AC. Vulnerable atherosclerotic plaque metalloproteinases and foam cell phenotypes. Thromb Haemost 2009 Jun;101(6):1006-1011.
(5) Newby AC. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. Physiol Rev 2005 Jan;85(1):1-31.
(6) Medbury HJ, James V, Ngo J, Hitos K, Wang Y, Harris DC, et al. Differing association of macrophage subsets with atherosclerotic plaque stability. Int Angiol 2013 Feb;32(1):74-84.
(7) Jager NA, Teteloshvili N, Zeebregts CJ, Westra J, Bijl M. Macrophage folate receptor beta (FR-beta) expression in auto-immune inflammatory rheumatic diseases: A forthcoming marker for cardiovascular risk? Autoimmun Rev 2011 Nov 7.
(8) Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004 Dec;25(12):677-686.
(9) Wolfs IM, Donners MM, de Winther MP. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost 2011 Nov;106(5):763-771.
(10) Raffetto JD, Khalil RA. Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. Biochem Pharmacol 2008 Jan 15;75(2):346-359.
(11) Loftus IM, Naylor AR, Goodall S, Crowther M, Jones L, Bell PR, et al. Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. Stroke 2000 Jan;31(1):40-47.
(12) Alvarez B, Ruiz C, Chacon P, Alvarez-Sabin J, Matas M. Serum values of metalloproteinase-2 and metalloproteinase-9 as related to unstable plaque and inflammatory cells in patients with greater than 70% carotid artery stenosis. J Vasc Surg 2004 Sep;40(3):469-475.
(13) Taurino M, Raffa S, Mastroddi M, Visco V, Rizzo L, Torrisi MR, et al. Metalloproteinase expression in carotid plaque and its correlation with plasma levels before and after carotid endarterectomy. Vasc Endovascular Surg 2007 Dec-2008 Jan;41(6):516-521.
(14) Sapienza P, di Marzo L, Borrelli V, Sterpetti AV, Mingoli A, Piagnerelli R, et al. Basic fibroblast growth factor mediates carotid plaque instability through metalloproteinase-2 and -9 expression. Eur J Vasc Endovasc Surg 2004 Jul;28(1):89-97.
(15) Kunte H, Amberger N, Busch MA, Ruckert RI, Meiners S, Harms L. Markers of instability in high-risk carotid plaques are reduced by statins. J Vasc Surg 2008 Mar;47(3):513-522.
(16) Eldrup N, Gronholdt ML, Sillesen H, Nordestgaard BG. Elevated matrix metalloproteinase-9 associated with stroke or cardiovascular death in patients with carotid stenosis. Circulation 2006 Oct 24;114(17):1847-1854.
(17) Turu MM, Krupinski J, Catena E, Rosell A, Montaner J, Rubio F, et al. Intraplaque MMP-8 levels are increased in asymptomatic patients with carotid plaque progression on ultrasound. Atherosclerosis 2006 Jul;187(1):161-169.
(18) Johnson JL, George SJ, Newby AC, Jackson CL. Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. Proc Natl Acad Sci U S A 2005 Oct 25;102(43):15575-15580.
(19) Wallis de Vries BM, van Dam GM, Tio RA, Hillebrands JL, Slart RH, Zeebregts CJ. Current imaging modalities to visualize vulnerability within the atherosclerotic carotid plaque. J Vasc Surg 2008 Dec;48(6):1620-1629.
(20) Sanz J, Fayad ZA. Imaging of atherosclerotic cardiovascular disease. Nature 2008 Feb 21;451(7181):953-957.

(21) Deguchi JO, Aikawa M, Tung CH, Aikawa E, Kim DE, Ntziachristos V, et al. Inflammation in atherosclerosis: visualizing matrix metalloproteinase action in macrophages in vivo. Circulation 2006 Jul 4;114(1):55-62.
(22) Jaffer FA, Kim DE, Quinti L, Tung CH, Aikawa E, Pande AN, et al. Optical visualization of cathepsin K activity in atherosclerosis with a novel, protease-activatable fluorescence sensor. Circulation 2007 May 1;115(17):2292-2298.
(23) Wallis de Vries BM, Hillebrands JL, van Dam GM, Tio RA, de Jong JS, Slart RH, et al. Images in cardiovascular medicine. Multispectral near-infrared fluorescence molecular imaging of matrix metalloproteinases in a human carotid plaque using a matrix-degrading metalloproteinase-sensitive activatable fluorescent probe. Circulation 2009 May 26;119(20):e534-6.
(24) Meerwaldt R, Hermus L, Reijnen MM, Zeebregts CJ. Carotid endarterectomy: current consensus and controversies. Surg Technol Int 2010 Oct;20:283-291.
(25) Jager NA, Westra J, van Dam GM, Teteloshvili N, Tio RA, Breek JC, et al. Targeted folate receptor beta fluorescence imaging as a measure of inflammation to estimate vulnerability within human atherosclerotic carotid plaque. J Nucl Med 2012 Aug;53(8):1222-1229.
(26) Jaffer FA, Vinegoni C, John MC, Aikawa E, Gold HK, Finn AV, et al. Real-time catheter molecular sensing of inflammation in proteolytically active atherosclerosis. Circulation 2008 Oct 28;118(18):1802-1809.
(27) Choudhary S, Higgins CL, Chen IY, Reardon M, Lawrie G, Vick GW,3rd, et al. Quantitation and localization of matrix metalloproteinases and their inhibitors in human carotid endarterectomy tissues. Arterioscler Thromb Vasc Biol 2006 Oct;26(10):2351-2358.
(28) Sluijter JP, Pulskens WP, Schoneveld AH, Velema E, Strijder CF, Moll F, et al. Matrix metalloproteinase 2 is associated with stable and matrix metalloproteinases 8 and 9 with vulnerable carotid atherosclerotic lesions: a study in human endarterectomy specimen pointing to a role for different extracellular matrix metalloproteinase inducer glycosylation forms. Stroke 2006 Jan;37(1):235-239.
(29) Verhoeven B, Hellings WE, Moll FL, de Vries JP, de Kleijn DP, de Bruin P, et al. Carotid atherosclerotic plaques in patients with transient ischemic attacks and stroke have unstable characteristics compared with plaques in asymptomatic and amaurosis fugax patients. J Vasc Surg 2005 Dec;42(6):1075-1081.
(30) Heo SH, Cho CH, Kim HO, Jo YH, Yoon KS, Lee JH, et al. Plaque rupture is a determinant of vascular events in carotid artery atherosclerotic disease: involvement of matrix metalloproteinases 2 and 9. J Clin Neurol 2011 Jun;7(2):69-76.
(31) Tziakas DN, Lazarides MK, Tentes IK, Georgiadis GS, Eleftheriadou E, Chalikias GK, et al. Gelatinases [matrix metalloproteinase-2 (MMP-2) and MMP-9] induce carotid plaque instability but their systemic levels are not predictive of local events. Ann Vasc Surg 2005 Jul;19(4):529-533.
(32) Zajac E, Schweighofer B, Kupriyanova TA, Juncker-Jensen A, Minder P, Quigley JP, et al. Angiogenic capacity of M1- and M2-polarized macrophages is determined by the levels of TIMP-1 complexed with their secreted proMMP-9. Blood 2013 Dec 12;122(25):4054-4067.

CHAPTER 9

Summary, discussion, conclusion and future perspectives



Summary and discussion

Atherosclerosis is an inflammatory disease of the vessel wall (1). The pathogenesis of atherosclerosis is, among others, associated with auto-immune inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Atherosclerosis is a chronic process that leads to the formation of atherosclerotic plaques. These plaques may become vulnerable and rupture, which is directly related to cardiovascular diseases (CVD) such as myocardial infarction or ischemic stroke. The development of an atherosclerotic plaque starts with an increased permeability of the endothelium. Subsequently, retention and accumulation of low-density lipoprotein (LDL) in the vessel wall, followed by endothelial activation and chemokine secretion occurs (2). In response, monocytes are attracted and migrate into the vessel wall where they differentiate into macrophages (3). Characteristics of a vulnerable atherosclerotic plaque (i.e. one prone to rupture) are inflammation (often defined as extensive macrophage accumulation), elevation of matrix metalloproteinases (MMPs), a thin fibrous cap with a large lipid core, superficial erosion and platelet aggregation or fibrin deposition, a fissured plaque cap and severe luminal stenosis (4). Potentially, macrophages can be used to visualize inflammatory responses in atherosclerosis.

Part I
Markers for early atherosclerosis

Endothelial progenitor cells

As stated, the development of an atherosclerotic plaque starts with changes in permeability of the endothelium. This results in the upregulation of several membrane proteins, as such considered as endothelial activation markers. In addition, endothelial dysfunction occurs, and this might be regarded as a first sign of the atherosclerotic process. To stop this process, recovery is required. Endothelial progenitor cells (EPCs) appear to be important in endothelial repair (5). EPCs can repair or form new blood vessels through two different mechanisms: by endothelial sprouting from a preexisting capillary network (angiogenesis) or by vasculogenesis, which refers to blood vessel formation from EPCs differentiating *in situ* (6). Therefore, number and functionality of circulating EPCs may be an important factor contributing to the recovery in the atherogenic process (7). In ***chapter 2***, two methods (flow cytometry and culture) to measure EPCs are used to study EPC counts in RA patients with newly onset disease (diagnosis ≤ 1.5 year) compared to healthy sex and age matched controls. RA patients had significantly lower endothelial repair cells. Furthermore, EPCs were inversely related to RA disease activity, which might suggest EPC shortage in peripheral blood is related to the accelerated development of atherosclerosis in RA patients with higher disease activity.

Endothelial activation markers

Endothelial cell activation through inflammatory vessel wall damage might be another reason for the increased prevalence of CVD due to atherosclerosis observed in RA patients, as well as in other autoimmune diseases (8). Endothelial cell activation is represented by the production of vascular endothelial growth factor (VEGF) and the release of angiopoietin-2 (ANGP-2), which antagonizes binding of angiopoietin -1 (ANGP-1) to the Tie-2 receptor. In ***chapter 2***, it is shown that VEGF serum levels are increased in RA patients. Furthermore, EPC numbers were inversely correlated to ANGP-2 levels. Endothelial cell activation sensitizes the endothelial cells to up-regulate and release adhesion molecules (9), such as soluble vascular cell adhesion molecule-1 (sVCAM-1), thrombomodulin (TM or CD141) and von Willebrand Factor (vWF). In ***chapter 3*** endothelial activation markers, advanced glycation end products (AGEs) and SAE were measured in RA patients early in the course of their disease, and subsequently after one year. Measurements were related to disease activity at both time points. Endothelial dysfunction was present in newly diagnosed RA patients, independently of traditional risk factors and was inversely correlated with disease activity. By reducing disease activity endothelial dysfunction improved, though not to normal values. In ***chapter 2*** and ***3*** we found sVCAM-1 and vWF to be increased in early RA patients compared to healthy controls in peripheral blood. Moreover, sVCAM-1 was inversely correlated to EPC counts, and sVCAM-1 had the strongest association with EPC count in multivariate analysis. This indicates that sVCAM-1 might be a good systemic marker for predicting cardiovascular risk in chronic inflammatory diseases (10).

Small arterial elasticity

Endothelial dysfunction results in increased 'stiffness' of the arterial wall. This can be measured using tonometry of the radial artery by pulse wave analysis (PWA), which is recalculated to Small Arterial Elasticity (SAE) (11). In ***chapter 3***, while SAE was decreased in RA patients versus healthy controls, we found SAE was inversely correlated with DAS-28. Of note, after one year disease activity was reduced and SAE significantly improved in RA patients. So by reducing disease activity in RA patients, endothelial dysfunction improves. In ***chapter 2*** SAE was inversely related to both methods of EPC measurement. This shows SAE might be a good marker for cardiovascular risk in patients with systemic disease during their disease.

Advanced glycation end products

Advanced glycation end products (AGEs) are stable structures accumulating on long-lived proteins, formed by non-enzymatic glycation and oxidative reactions (12). By interaction with their major receptor (RAGE), AGEs have been shown *in vitro* to promote inflammatory mechanisms leading to atherosclerotic plaque formation and vulnerability (13). AGEs accumulate in tissues with slow turnover such as the vessel wall, cartilage and skin, and can be measured by evaluating skin autofluorescence (skin AF) (14). In ***chapter 3***, AGEs

were shown to be significantly increased in RA patients versus healthy controls. In healthy controls without cardiovascular diseases, but with a wide range of Framingham risk scores (10 year risk on coronary heart disease (i.e. myocardial infarction or death from coronary heart disease)), a relation with skin AF and traditional risk factors for atherosclerosis (age, obesity, BMI, presence of DM and plasma glucose levels) was found and described in ***chapter 4***. Also, skin AF was positively associated with plasma levels of matrix metalloproteinases (MMP)-3, 9, tissue inhibitor of metalloproteinases (TIMP)-1 and hsCRP, Framingham risk score (15), plaque score and IMT in subjects without cardiovascular disease. As such, AGEs measured by skin AF might be useful for use as clinical biomarker for subclinical atherosclerosis in risk groups such as diabetes mellitus (DM) or patients with autoimmune inflammatory disease.

Part II
Imaging of atherosclerosis

Identification of the vulnerable plaque is a major challenge in cardiovascular medicine, as conventional imaging technologies such as B-mode duplex ultrasound, CT angiography scanning and magnetic resonance imaging (MRI) used in the clinic, offer minimal information about the underlying biology and potential risk for rupture (16). So the conventional diagnostic imaging methods do not provide information about plaque vulnerability (presence of lipid pools and necrotic core with an overlying thin fibrous cap, increased macrophage activity and plaque progression). In addition, characteristics of a vulnerable plaque are observed within plaques that are modest in terms of angiographic stenosis severity (17). Molecular imaging has the potential to visualize functional biological processes and, as such, plaque vulnerability. Until now, lipid accumulation, apoptosis and calcification, angiogenesis, thrombosis, proteolysis and inflammation have been targets for innovative imaging techniques (18). Targets that may be most useful for clinical imaging applications are inflammatory cells or their products (19). Imaging inflammation using folate receptor-β (FR-β; a 38-kDa glycosylphosphatidylinositol-anchored protein that binds with high affinity to vitamin folic acid (20)) is a very promising tool to identify vulnerable plaques in early diagnosis of vascular diseases in association with systemic diseases (***chapter 5***), as FR-ß is selectively expressed on activated macrophages due to an increase in DNA syntheses of various proteins (21). Three isoforms of folate receptor have been identified; an alpha (FR-α), beta (FR-ß) and gamma isoform (FR-γ). FR-α is overexpressed on tumor cells in specific types of cancer, while FR-γ is a secreted protein from hematopoietic cells. To investigate usefulness of different imaging modalities these were tested ex-vivo in plaques of atherosclerotic patients, and validated for specificity with immunohistochemical staining and realtime PCR. In ***chapter 6*** *ex vivo* optical imaging of FR-β with an optical fluorescent contrast agent (FITc) in atherosclerotic plaques from the carotid artery was described. Compared to areas with low folate–FITc uptake, areas of high folate–FITc uptake within human atherosclerotic plaques had an increased number of activated macrophages and higher areas of hypoxia, as shown by higher expression if HIF-1α (22). These characteristics of vulnerability imply that molecular imaging of FR-β through folate conjugates might be a good indicator for plaque vulnerability in future noninvasive imaging studies. MMPs are produced by activated macrophages and play a role in destabilizing the atherosclerotic plaque. In ***chapter 8***, we found MMP-9 is the most dominantly MMP present in atherosclerotic plaques and is produced by M2 rather than M1 macrophages. MMPs were targeted with a MMP-sensitive probe (MMPsense) and visualized by fluorescence imaging. MMPsense is activated by MMP -2, -3, -9 and -13. It is optically silent in its inactivated state and becomes highly fluorescent following protease-mediated activation. MMPsense can therefore be used to detect MMP-9 in plaques and might be a good marker to investigate plaque instability in the clinical setting. However, in

the *in vivo* situation, penetration depth of fluorescence imaging is limited. Therefore, the use of radiolabeled folate (^{99m}Tc-folate) described in ***chapter 7*** could be of more clinical value.

Macrophage plasticity

In human atherosclerotic lesions, a heterogeneous population of macrophages exists; a classically activated macrophage type (M1) as well as an alternatively activated macrophage population (M2) (23). The M1 macrophage is thought to have pro-inflammatory properties, promotes lesion development, and is driven by lipopolysaccharide (LPS) and interferon gamma (IFN-γ). M2 macrophages however, are anti-inflammatory and immune regulatory. Upon cytokine stimulation they modify the development of atherosclerotic plaques. The M2 macrophage population can be divided in M2a, M2b and M2c subtypes, depending on the cytokine environment (IL-4, immune complexes and IL-10 respectively) (24). As the function of M2a macrophages is type II inflammation (wound-healing or tissue-repair functions), and type M2c matrix deposition and tissue remodeling; the type of macrophage present might be most important in atherosclerosis (25). However, the situation *in vivo* is thought to be rather more complex than the typically described M1 and M2 macrophages distribution. That is, not only cytokine environment, but also other factors could play a major role (26). In ***chapter 7*** the use of ^{99m}Tc-folate to discriminate between an M1-like and M2-like macrophage phenotype within an atherosclerotic carotid plaque is described. We found more M2-like macrophages in areas of high ^{99m}Tc-folate accumulation, compared to areas with low accumulation of the folate probe, suggesting that FR-ß is expressed at a higher level on M2 macrophages. In ***chapter 8***, macrophages were differentiated and polarized *in vitro* into M1, M2a and M2c macrophages to investigate expression of MMPs in these subtypes, as well is in smooth muscle cells (SMCs). These experiments showed M2 macrophages had higher expression of MMP-9, while MMP-2 was higher expressed in M1 macrophages and SMCs.

Conclusion

The prevalence of CVD due to atherosclerosis is increased in RA patients, as well as in other autoimmune-immune inflammatory diseases. The pathogenesis of systemic autoimmune diseases and atherosclerosis underlying CVD are very alike but complex. We examined markers for different stages of the accelerated atherosclerotic process occurring in autoimmune mediated patients. Also we found the impact of traditional (such as BMI, smoking) and non-traditional (disease activity) risk factors both play a role in the development of atherosclerosis. In **part I**, systemic markers for early atherosclerosis were assessed, and compared to conventional measurements for atherosclerosis such as IMT. EPC count might be a promising systemic marker for subclinical atherosclerosis, as EPC count is inversely related to disease activity, which in turn seems to be related to accelerated

atherosclerosis in early RA patients. However, a universal protocol for measuring EPC counts in peripheral blood is still lacking. Therefore, EPC measurement combined with sVCAM-1 and non invasive measurement of AGEs and SAE gives a better overview of the cardiovascular status of patients at risk for atherosclerosis. Furthermore, measurement of AGEs and SAE is simple and application in a clinical setting can easily be done. In **part II**, new and existing imaging techniques were used to examine probes that make inflammation within a carotid plaque visible. The ultimate goal was discrimination between vulnerable, risk-full atherosclerotic plaques that could cause symptoms, and the more stable ones. Compared with areas with low folate uptake, areas of high folate uptake within human atherosclerotic plaques had an increased number of activated macrophages. Several techniques (flow cytometry, immuno- and fluorescence staining) showed FR-β was over expressed on M2 macrophages. Although both FR-β imaging and MMP-imaging showed that activated macrophages were targeted, more research is needed to discriminate which mechanisms play a role in the process of a plaque becoming vulnerable, to select for patients eligible for treatment from those with a low risk. However, using SPECT ^{99m}Tc-folate imaging as a marker for M2-like macrophages in atherosclerosis is a promising tool for plaque vulnerability and therefore could potentially be used in the diagnostic process. Measuring MMPs in serum or using fluorescence or radiolabeled imaging for plaques could be promising as well, since MMPs are directly involved in degradation of strength-giving collagens and other structural proteins of the arterial extracellular matrix. Overproduction of MMPs by macrophages could therefore promote atherosclerotic plaque rupture (27). Of all the MMPs studied in this thesis, MMP-9 was the best detectable using RT-PCR within an atherosclerotic carotid plaque. Moreover, MMP-9 can be detected using fluorescence (MMPsense), high-resolution deep-tissue multispectral optoacoustic tomography (MSOT) technology (28), or using radiolabeled probes. Still more research is needed which MMPs are the most important in the process of a plaque becoming vulnerable and their expression among macrophage phenotypes.

Future perspectives

Tracers targeting factors contributing to plaque vulnerability such as inflammation and proteolysis will help to get a clear view of atherosclerotic disease *in vivo* in the near future. As a result, this accelerates the individualization of the diagnostical process. Ultimately, this will avoid plaque rupture leading to a decrease of acute coronary events and decreased mortality. However, obstacles with tracer specificity and sensitivity of imaging technologies have to be overcome. Firstly, more specific probes targeting distinct cellular processes have to be found. Secondly, the detection technologies have to become more sensitive. MRI and computed tomography (CT) have a good spatial resolution, but a low sensitivity. Optical techniques, such as bioluminescence or fluorescence imaging, have restricted tissue penetration depth, presenting challenges to imaging deep arteries in large animals

and humans. Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) stand out because of their high sensitivity for small amounts of probe mass, but these modalities have relatively limited spatial resolution (4). Imaging via the intravascular route may have the potential to further improve these imaging technologies (19). In that manner, the cardiovascular imaging may benefit from cancer research, where implication of biomarkers in daily patient care seems feasible (29). Experimental investigations have used these techniques in conjunction with a variety of molecular-targeted compounds, but few have made their way into the clinic (30). Also, testing of toxicity and pharmacokinetics still needs to be performed for most of the tracers. Access to a cyclotron and radio pharmacy facilities for production of some of the above named molecular probes is needed. While often available in specialized centres, the infrastructure for advanced imaging may limit wide-spread implementation (31). Although costs of new imaging technologies are quite high, these may be modest compared to the general healthcare costs to treat patients with vascular complications. Fluorescence imaging using a folate–FITC optical contrast agent probe can visualize the distribution of activated macrophages and is therefore a promising marker of inflammation and vulnerability of human atherosclerotic plaques. Folate has already been used in humans to identify tumor processes, because several solid tumors express FR-α (29). However, current applications for noninvasive optical imaging of fluorescent signals within a human carotid artery are limited by the use of fluorophores with emission wavelengths within the range of 450–600 nm such as FITC, leading to a limited penetration depth. Therefore, sophisticated imaging devices such as multispectral optoacoustic tomography for imaging of near-infrared fluorophores (e.g., IRDye-CW800) conjugated to folate might solve this problem for future clinical applications. Another promising tool would be targeting the mannose receptor (expressed by M2-like macrophages) by 2-deoxy-2-[(18)F]fluoro-D-mannose, ([(18)F]FDM) imaging (32). Also the tracer MTHPT (C6H12O5) therefore is an option, since MTHPT has the advantage over other tracers for this purpose as it shows a higher selectivity towards macrophages. MTHPT is a ligand for mannose receptor (MR), but has shown – unlike other tracers – no (or very low) affinity for glucose transporter proteins (GLUTs). Next to imaging, measuring systemic markers would be beneficial to the diagnostic process in patients at risk for (sub) clinical atherosclerosis. As the most promising markers named in the conclusion (EPC measurement combined with sVCAM-1 and non invasive measurement of AGEs and SAE) all have their advantages and disadvantages, longitudinal studies combining different markers would be recommended. Since EPC count is inversely related to disease activity, one hypothesis could be endothelial activation, dysfunction and accelerated atherosclerosis occur more during episodes of active disease in RA patients. However, more studies are needed to further unravel the pathogenic mechanism behind this shared pathology and to find the biomarker which best reflects the atherosclerotic risk in autoimmune-immune inflammatory disease patients.

Taken together, measuring systemic markers (early disease stage) and imaging subclinical atherosclerosis (later stage) may improve risk stratification for the development of CVD. As such, improving current prediction models and developing targets for specific therapeutic interventions for autoimmune-immune inflammatory disease patients becomes a possibility.

References

(1) Libby P. Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol 2012 Sep;32(9):2045-2051.
(2) Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011 May 19;473(7347):317-325.
(3) Stoger JL, Goossens P, de Winther MP. Macrophage heterogeneity: relevance and functional implications in atherosclerosis. Curr Vasc Pharmacol 2010 Mar;8(2):233-248.
(4) Camici PG, Rimoldi OE, Gaemperli O, Libby P. Non-invasive anatomic and functional imaging of vascular inflammation and unstable plaque. Eur Heart J 2012 Jun;33(11):1309-1317.
(5) Hristov M, Zernecke A, Liehn EA, Weber C. Regulation of endothelial progenitor cell homing after arterial injury. Thromb Haemost 2007 Aug;98(2):274-277.
(6) Tepper OM, Galiano RD, Kalka C, Gurtner GC. Endothelial progenitor cells: the promise of vascular stem cells for plastic surgery. Plast Reconstr Surg 2003 Feb;111(2):846-854.
(7) Rosenzweig A. Circulating endothelial progenitors--cells as biomarkers. N Engl J Med 2005 Sep 8;353(10):1055-1057.
(8) Bijl M. Endothelial activation, endothelial dysfunction and premature atherosclerosis in systemic autoimmune diseases. Neth J Med 2003 Sep;61(9):273-277.
(9) Korff T, Ernst E, Nobiling R, Feldner A, Reiss Y, Plate KH, et al. Angiopoietin-1 mediates inhibition of hypertension-induced release of angiopoietin-2 from endothelial cells. Cardiovasc Res 2012 Jun 1;94(3):510-518.
(10) Constans J, Conri C. Circulating markers of endothelial function in cardiovascular disease. Clin Chim Acta 2006 Jun;368(1-2):33-47.
(11) Van Doornum S, McColl G, Jenkins A, Green DJ, Wicks IP. Screening for atherosclerosis in patients with rheumatoid arthritis: comparison of two in vivo tests of vascular function. Arthritis Rheum 2003 Jan;48(1):72-80.
(12) Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. Circulation 2006 Aug 8;114(6):597-605.
(13) Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, et al. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med (Berl) 2005 Nov;83(11):876-886.
(14) Meerwaldt R, Graaff R, Oomen PH, Links TP, Jager JJ, Alderson NL, et al. Simple non-invasive assessment of advanced glycation endproduct accumulation. Diabetologia 2004 Jul;47(7):1324-1330.
(15) Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. Circulation 1998 May 12;97(18):1837-1847.
(16) Wallis de Vries BM, van Dam GM, Tio RA, Hillebrands JL, Slart RH, Zeebregts CJ. Current imaging modalities to visualize vulnerability within the atherosclerotic carotid plaque. J Vasc Surg 2008 Dec;48(6):1620-1629.
(17) Puri R, Worthley MI, Nicholls SJ. Intravascular imaging of vulnerable coronary plaque: current and future concepts. Nat Rev Cardiol 2011 Mar;8(3):131-139.
(18) Hermus L, van Dam GM, Zeebregts CJ. Advanced carotid plaque imaging. Eur J Vasc Endovasc Surg 2010 Feb;39(2):125-133.
(19) Wildgruber M, Swirski FK, Zernecke A. Molecular imaging of inflammation in atherosclerosis. Theranostics 2013 Nov 1;3(11):865-884.
(20) Paulos CM, Turk MJ, Breur GJ, Low PS. Folate receptor-mediated targeting of therapeutic and imaging agents to activated macrophages in rheumatoid arthritis. Adv Drug Deliv Rev 2004 Apr 29;56(8):1205-1217.
(21) van der Heijden JW, Oerlemans R, Dijkmans BA, Qi H, van der Laken CJ, Lems WF, et al. Folate receptor beta as a potential delivery route for novel folate antagonists to macrophages in the synovial tissue of rheumatoid arthritis patients. Arthritis Rheum 2009 Jan;60(1):12-21.

(22) Sluimer JC, Gasc JM, van Wanroij JL, Kisters N, Groeneweg M, Sollewijn Gelpke MD, et al. Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. J Am Coll Cardiol 2008 Apr 1;51(13):1258-1265.
(23) Medbury HJ, James V, Ngo J, Hitos K, Wang Y, Harris DC, et al. Differing association of macrophage subsets with atherosclerotic plaque stability. Int Angiol 2013 Feb;32(1):74-84.
(24) Jager NA, Teteloshvili N, Zeebregts CJ, Westra J, Bijl M. Macrophage folate receptor beta (FR-beta) expression in auto-immune inflammatory rheumatic diseases: A forthcoming marker for cardiovascular risk? Autoimmun Rev 2011 Nov 7.
(25) Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004 Dec;25(12):677-686.
(26) Wolfs IM, Donners MM, de Winther MP. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost 2011 Nov;106(5):763-771.
(27) Newby AC. Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability. Arterioscler Thromb Vasc Biol 2008 Dec;28(12):2108-2114.
(28) Razansky D, Harlaar NJ, Hillebrands JL, Taruttis A, Herzog E, Zeebregts CJ, et al. Multispectral Optoacoustic Tomography of Matrix Metalloproteinase Activity in Vulnerable Human Carotid Plaques. Mol Imaging Biol 2011 Jul 1.
(29) van Dam GM, Themelis G, Crane LM, Harlaar NJ, Pleijhuis RG, Kelder W, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-alpha targeting: first in-human results. Nat Med 2011 Sep 18.
(30) Rudd JH, Hyafil F, Fayad ZA. Inflammation imaging in atherosclerosis. Arterioscler Thromb Vasc Biol 2009 Jul;29(7):1009-1016.
(31) Camici PG, Rimoldi OE, Gaemperli O, Libby P. Non-invasive anatomic and functional imaging of vascular inflammation and unstable plaque. Eur Heart J 2012 Jun;33(11):1309-1317.
(32) Tahara N, Mukherjee J, de Haas HJ, Petrov AD, Tawakol A, Haider N, et al. 2-deoxy-2-[(18)F]fluoro-d-mannose positron emission tomography imaging in atherosclerosis. Nat Med 2014 Feb;20(2):215-219.

NEDERLANDSE SAMENVATTING

Nederlandse Samenvatting

Achtergrond

Slagaderverkalking (atherosclerose) is een ontstekingsziekte van de vaatwand (1). Het ontstaan van atherosclerose is onder andere geassocieerd met auto-immuun ziekten zoals reumatoïde artritis (RA). Auto-immuun ziekten zijn ziekten waarbij het immuunsysteem zich tegen bestanddelen van het eigen lichaam keert. Atherosclerose is een chronisch proces dat leidt tot de vorming van atherosclerotische plaques, waardoor de vaten dunner worden. De ontwikkeling van een atherosclerotische plaque begint met een verhoogde doorlaatbaarheid van het endotheel (bekleding van een bloedvat), waardoor het 'slechte' cholesterol (LDL) door de endotheel laag heen kan en zich in de vaatwand kan opstapelen. Vervolgens worden de endotheel cellen geactiveerd (2) en gaan monocyten (een soort ontstekingscel) door de vaatwand heen, waar ze in macrofagen (letterlijk: veelvraten) veranderen (3). Als plaques kwetsbaar worden en scheuren kunnen hart -en vaatziekten ontstaan, zoals een hartinfarct of een beroerte. Kenmerken van een kwetsbare atherosclerotische plaque zijn: ontsteking, verhoging van eiwitten die de vaatwand kunnen afbreken, en ernstige vernauwing van een bloedvat (stenose) (4). Mogelijk zouden macrofagen gebruikt kunnen worden om ontstekingsreacties in atherosclerose in beeld te brengen, om zo de kans op hart- en vaatziekten te voorspellen.

Deel I - Markers voor vroege atherosclerose

Endotheliale voorlopercellen

De ontwikkeling van een atherosclerotische plaque een verhoogde doorlaatbaarheid van het endotheel. Dit resulteert in de activatie van verschillende membraaneiwitten, ook wel endotheliale activatie markers genoemd. Vervolgens treedt er endotheel disfunctie op. Dit kan als een eerste teken van het atherosclerotische proces worden beschouwd. Om dit proces te stoppen, is herstel nodig. Endotheliale voorloper cellen (EPCs) lijken belangrijk te zijn bij dit proces (5). EPCs kunnen nieuwe bloedvaten herstellen of vormen (6). Daarom kan het aantal en de functionaliteit van EPCs in het bloed een belangrijke factor zijn in het herstel proces van de vaten (7). In hoofdstuk 2 zijn twee methoden om EPCs te meten gebruikt. Daarbij wordt gekeken naar RA patiënten met een ziekte duur van minder dan 1,5 jaar. Deze data zijn vergeleken met gezonde vrijwilligers van hetzelfde geslacht en leeftijd. Daaruit bleek dat RA patiënten een significant lager aantal endotheliale voorloper cellen in hun bloed hebben. Bovendien is het aantal EPCs omgekeerd evenredig met de ziekteactiviteit van RA patiënten (gemeten door middel van de DAS-28 score). Dit suggereert dat EPC tekort in het perifere bloed gerelateerd is aan de versnelde ontwikkeling van atherosclerose bij RA patiënten met hogere ziekteactiviteit.

Endotheel activatie markers

De activering van endotheelcellen als gevolg van ontsteking kan een andere reden zijn voor het verhoogde voorkomen van hart- en vaatziekten bij RA patiënten (8). Endotheelcel activatie wordt gevolgd door de productie van vasculaire endotheliale groeifactoren (VEGF) wat ervoor zorgt dat verschillende eiwitten vrijkomen, zoals angiopoietine-2 (ANGP-2). ANGP-2 gaat de binding van angiopoietine -1 (ANGP-1) aan de Tie-2-receptor tegen (welke vaatnieuwvorming stimuleert). In hoofdstuk 2 wordt aangetoond dat VEGF serum spiegels verhoogd zijn bij RA patiënten. Ook zijn EPC aantallen omgekeerd evenredig aan het ANGP-2 niveau. Endotheelcel activatie wordt gekenmerkt door de productie van adhesiemoleculen (9), zoals het oplosbare vasculaire cel adhesie molecul-1 (sVCAM-1), trombomoduline (TM of CD141) en von Willebrand Factor (vWF). In hoofdstuk 3 worden endotheliale activatie markers, versuikerde eiwitten (zogenaamde advanced glycation end products (AGE's)) en de elasticiteit van kleine vaten (small arterial elasticity (SAE)) gemeten bij RA patiënten vroeg in het verloop van hun ziekte, en vervolgens na een jaar. Deze metingen zijn gerelateerd aan de ziekte activiteit op beide tijdstippen. Endotheliale disfunctie is aanwezig bij nieuw gediagnosticeerde RA patiënten, onafhankelijk van traditionele risicofactoren, en is omgekeerd gecorreleerd aan de ziekteactiviteit. Door het verminderen van ziekteactiviteit verbeterd de endotheel functie, maar niet tot normale waarden. In hoofdstuk 2 en 3 worden verhoogde sVCAM-1 en vWF waardes in het perifere bloed van vroege RA patiënten gevonden, vergeleken met gezonde controles. Bovendien is sVCAM-1 omgekeerd geassocieerd aan EPCs. sVCAM-1 heeft de sterkste associatie met EPCs in de multivariaat analyse. Dit geeft aan dat sVCAM-1 een goede voorspeller voor het cardiovasculaire risico bij chronische auto-immuunziekten is (10).

Elasticiteit van de kleine vaten

Endotheliale disfunctie resulteert in verhoogde 'stijfheid' van de vaatwand. Dit kan gemeten worden met behulp van het meten van de vorm van de drukgolf die zich bij elke hartslag door de slagader verspreidt, de zogenaamde 'pulse wave analyse (PWA)', die wordt herleid naar de elasticiteit van de kleine vaten (SAE) (11). In hoofdstuk 3 is beschreven dat de SAE afgenomen is bij RA patiënten versus gezonde controles, en dat de SAE omgekeerd gecorreleerd is met de ziekteactiviteit. Nadat de ziekteactiviteit na een jaar verminderd is, is ook de SAE significant verbeterd bij patiënten met RA. Dus door het verminderen van ziekteactiviteit bij RA-patiënten is de endotheliale functie ook beter. In hoofdstuk 2 is SAE omgekeerd evenredig aan beide methoden om EPCs te meten. Dit is een aanwijzing dat SAE een goede maat voor het risico op hart- en vaatziekten bij patiënten met een auto-immuun ziekte zou kunnen zijn.

Advanced glycation end products

AGEs zijn eiwitten die onherstelbaar beschadigd zijn, doordat er suikergroepen (zoals glucose) aan verankerd zijn en het eiwit is gedegenereerd (12). Door interactie met hun belan-

grijkste receptor (RAGE), leiden ze tot vorming van atherosclerotische plaques. Ook bevorderen AGEs kwetsbaarheid van een plaque (13). AGEs stapelen makkelijker in weefsels met trage celdeling zoals de vaatwand, kraakbeen en huid. AGEs kunnen gemeten worden door de huid met fluorescentie (skin AF) (14).In hoofdstuk 3 is aangetoond dat AGES aanzienlijk verhoogd zijn bij RA patiënten, vergeleken met gezonde controles. In gezonde controles zonder hart- en vaatziekten, is het risico op het ontstaan van deze ziekten (gemeten met de Framingham risicoscore), gerelateerd aan AGEs gemeten in de huid. Ook traditionele risicofactoren voor atherosclerose zoals bijvoorbeeld leeftijd en verhoogd BMI, zijn gerelateerd aan AGES gemeten in de huid. In hoofdstuk 4 wordt de relatie tussen AGES gemeten in de huid en spiegels van eerder genoemde MMP eiwitten getoond (15). Ook het aantal plaques aanwezig in de halsslagaders, en de IMT (mate van vernauwing in een vat) bij personen zonder hart- en vaatziekten zijn gerelateerd aan AGEs. Als zodanig kan AGEs gemeten in de huid als een bruikbare marker voor vroege atherosclerose in risicogroepen zoals diabetes mellitus (DM) of patiënten met auto-immuunziekte gezien worden.

Deel II – Atherosclerose in beeld

Identificatie van een kwetsbare plaque wordt gezien als een uitdaging in de cardiovasculaire geneeskunde. Beeldvormende technieken die in de kliniek gebruikt worden (duplex echografie, CT-angiografie scans en magnetische resonantie imaging (MRI)), laten de vernauwingsgraad zien, maar geven geen informatie over de mate van kwetsbaarheid van een plaque (16). Bovendien zijn de plaques met lage vernauwingsgraad vaak kwetsbaar (17). Moleculaire beeldvorming heeft de potentie om biologische processen en plaque kwetsbaarheid als zodanig te visualiseren. Tot nu toe zijn er verschillende biologische processen in beeld gebracht (18). Doelen die het meest nuttig zijn voor klinische beeldvorming zijn ontstekingscellen of hun producten (19), omdat deze een direct relatie met kwetsbaarheid hebben. Folaat receptor-β (FR-β) is een eiwit dat zich op de oppervlakte van macrofagen bevindt (20). Het is een veelbelovend middel om kwetsbare plaques in een vroeg stadium te diagnosticeren (hoofdstuk 5). In hoofdstuk 6 is het in beeld brengen van FR-β met een optisch fluorescerend contrastmiddel (FITC) in atherosclerotische plaques uit de halsslagader beschreven. Vergeleken met gebieden met lage folaat-FITC opname, hadden gebieden met een hoge folaat-FITC opname in humane atherosclerotische plaques meer kenmerken van ontsteking en dus kwetsbaarheid (21). Dit impliceert dat moleculaire beeldvorming van FR-β een goede indicator voor plaque instabiliteit in de toekomst zou kunnen zijn. Een andere manier om instabiele plaques op te sporen is het in beeld brengen van MMPs. MMPs zijn eiwitten die de matrix afbreken, en de plaque destabiliseren. Ze worden geproduceerd door macrofagen. Er zijn verschillende MMP types. In hoofdstuk 8 vonden we dat MMP-9 het meest dominant aanwezig was in atherosclerotische plaques.

Macrofaag plasticiteit

In humane atherosclerotische plaques bestaat een heterogene populatie van macrofagen; het klassiek geactiveerd macrofaag type (M1) en een alternatief geactiveerde macrofaag populatie (M2) (22). De M1 macrofaag wordt gedacht pro-inflammatoire eigenschappen te hebben. De M1 macrofaag wordt aangedreven door lipopolysaccharide (LPS) en interferon gamma (IFN-γ). M2 macrofagen hebben een meer immuun regulerende werking. De M2 macrofaag populatie kan worden onderverdeeld in M2a, M2b en M2c subtypes, afhankelijk van het cytokine milieu (cytokinen activeren immuuncellen) (23). M2a en M2c zijn het belangrijkste in het atherosclerotische proces (24). De functie van M2a macrofagen is type II ontsteking (wondgenezing en/of het repareren van weefsel), de functie van type M2c macrofagen is matrix depositie en weefsel remodellering. MMP-9 wordt geproduceerd door M2 macrofagen (alternatief geactiveerd), en in mindere mate door M1 macrofagen (geactiveerd via de klassieke route). MMPs zijn in beeld gebracht met behulp van het fluorescerende stofje MMPsense. MMPsense wordt geactiveerd door MMP -2, -3, -9 en -13. MMPsense kan derhalve worden gebruikt om MMP-9 te detecteren en zou daarom een goede marker voor plaque instabiliteit in de kliniek kunnen zijn. Echter, in een menselijk lichaam kan de waarde van fluorescentie beeldvorming beperkt zijn vanwege de lage penetratie diepte. Daarom kan het gebruik van radioactief gelabeld folaat (^{99m}Tc-folaat) beschreven in hoofdstuk 7 van meer klinische waarde zijn. De situatie in het lichaam zou echter ingewikkelder kunnen zijn dan de verdeling in alléén M1 en M2 macrofagen. Niet alleen cytokine milieu, maar ook andere factoren kunnen een belangrijke rol spelen (25). In hoofdstuk 7 wordt door het gebruik van ^{99m}Tc-folaat onderscheid gemaakt tussen een M1-achtig en M2-achtig macrofaag fenotype binnen een atherosclerotische plaque. Er zijn meer M2-achtige macrofagen gevonden in gebieden met een hoge ^{99m}Tc-folaat accumulatie, wat suggereert dat FR-ß meer tot expressie komt op M2 macrofagen dan op M1 macrofagen. In hoofdstuk 8 worden macrofagen gedifferentieerd en gepolariseerd in het lab in M1, M2a en M2c macrofagen. Expressie van MMPs in deze subtypes zijn onderzocht, alsook in gladde spiercellen (SMC). Deze experimenten toonden dat M2 macrofagen hogere expressie van MMP-9 hadden, terwijl MMP-2 meer op M1 macrofagen en gladde spiercellen tot expressie werd gebracht.

Referenties

(1) Libby P. Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol 2012 Sep;32(9):2045-2051.
(2) Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011 May 19;473(7347):317-325.
(3) Stoger JL, Goossens P, de Winther MP. Macrophage heterogeneity: relevance and functional implications in atherosclerosis. Curr Vasc Pharmacol 2010 Mar;8(2):233-248.
(4) Camici PG, Rimoldi OE, Gaemperli O, Libby P. Non-invasive anatomic and functional imaging of vascular inflammation and unstable plaque. Eur Heart J 2012 Jun;33(11):1309-1317.
(5) Hristov M, Zernecke A, Liehn EA, Weber C. Regulation of endothelial progenitor cell homing after arterial injury. Thromb Haemost 2007 Aug;98(2):274-277.
(6) Tepper OM, Galiano RD, Kalka C, Gurtner GC. Endothelial progenitor cells: the promise of vascular stem cells for plastic surgery. Plast Reconstr Surg 2003 Feb;111(2):846-854.
(7) Rosenzweig A. Circulating endothelial progenitors--cells as biomarkers. N Engl J Med 2005 Sep 8;353(10):1055-1057.
(8) Bijl M. Endothelial activation, endothelial dysfunction and premature atherosclerosis in systemic autoimmune diseases. Neth J Med 2003 Sep;61(9):273-277.
(9) Korff T, Ernst E, Nobiling R, Feldner A, Reiss Y, Plate KH, et al. Angiopoietin-1 mediates inhibition of hypertension-induced release of angiopoietin-2 from endothelial cells. Cardiovasc Res 2012 Jun 1;94(3):510-518.
(10) Constans J, Conri C. Circulating markers of endothelial function in cardiovascular disease. Clin Chim Acta 2006 Jun;368(1-2):33-47.
(11) Van Doornum S, McColl G, Jenkins A, Green DJ, Wicks IP. Screening for atherosclerosis in patients with rheumatoid arthritis: comparison of two in vivo tests of vascular function. Arthritis Rheum 2003 Jan;48(1):72-80.
(12) Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. Circulation 2006 Aug 8;114(6):597-605.
(13) Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, et al. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med (Berl) 2005 Nov;83(11):876-886.
(14) Meerwaldt R, Graaff R, Oomen PH, Links TP, Jager JJ, Alderson NL, et al. Simple non-invasive assessment of advanced glycation endproduct accumulation. Diabetologia 2004 Jul;47(7):1324-1330.
(15) Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. Circulation 1998 May 12;97(18):1837-1847.
(16) Wallis de Vries BM, van Dam GM, Tio RA, Hillebrands JL, Slart RH, Zeebregts CJ. Current imaging modalities to visualize vulnerability within the atherosclerotic carotid plaque. J Vasc Surg 2008 Dec;48(6):1620-1629.
(17) Puri R, Worthley MI, Nicholls SJ. Intravascular imaging of vulnerable coronary plaque: current and future concepts. Nat Rev Cardiol 2011 Mar;8(3):131-139.
(18) Hermus L, van Dam GM, Zeebregts CJ. Advanced carotid plaque imaging. Eur J Vasc Endovasc Surg 2010 Feb;39(2):125-133.
(19) Wildgruber M, Swirski FK, Zernecke A. Molecular imaging of inflammation in atherosclerosis. Theranostics 2013 Nov 1;3(11):865-884.
(20) Paulos CM, Turk MJ, Breur GJ, Low PS. Folate receptor-mediated targeting of therapeutic and imaging agents to activated macrophages in rheumatoid arthritis. Adv Drug Deliv Rev 2004 Apr 29;56(8):1205-1217.
(21) Sluimer JC, Gasc JM, van Wanroij JL, Kisters N, Groeneweg M, Sollewijn Gelpke MD, et al. Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. J Am Coll Cardiol 2008 Apr 1;51(13):1258-1265.

(22) Medbury HJ, James V, Ngo J, Hitos K, Wang Y, Harris DC, et al. Differing association of macrophage subsets with atherosclerotic plaque stability. Int Angiol 2013 Feb;32(1):74-84.
(23) Jager NA, Teteloshvili N, Zeebregts CJ, Westra J, Bijl M. Macrophage folate receptor beta (FR-beta) expression in auto-immune inflammatory rheumatic diseases: A forthcoming marker for cardiovascular risk? Autoimmun Rev 2011 Nov 7.
(24) Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004 Dec;25(12):677-686.
(25) Wolfs IM, Donners MM, de Winther MP. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost 2011 Nov;106(5):763-771.

DANKWOORD

Dankwoord

Vanwege praktische redenen heb ik in 2008 besloten mijn wetenschapsstage vóór mijn coschappen te doen, wat normaal gesproken andersom is. Met de achterliggende gedachte: dan kan ik zo snel mogelijk door met datgene waarvoor ik met de opleiding geneeskunde begonnen ben. Echter, het werk op het lab beviel me veel beter dan gedacht. Zelfs zo goed, dat ik besloot te solliciteren voor een MD/PhD traject. Nu ik met gepaste trots het resultaat in handen heb, denk ik toch vooral aan hoeveel ik geleerd heb tijdens de totstandkoming van dit proefschrift. Of zoals Henry Miller zei: "one's destination is never a place, but a new way of looking at things." Ik wil dan ook iedereen die bij de weg naar deze bestemming betrokken is geweest bedanken, waarvan een aantal mensen in het bijzonder.

Ten eerste mijn copromotoren, Hannie Westra, Marc Bijl en Hendrikus Boersma, en mijn promotor Clark Zeebregts.

Hannie, jij bent de motor achter dit project geweest, en ik durf met zekerheid te zeggen dat zonder jou dit proefschrift niet op deze manier tot stand zou zijn gekomen. Bedankt dat ik altijd op jou kan rekenen, bij jou binnen kon lopen voor vragen, en mocht leren van jou uitgebreide kennis wat betreft het labwerk. Ook al heb je veel andere AIO's en studenten onder je hoede, er was altijd tijd voor overleg. Ik heb onze samenwerking als zeer prettig ervaren en ontzettend veel van jou geleerd.

Marc, aan jou kritische blik heb ik veel gehad. Vooral het lezen en schrijven van manuscripten –duidelijk en zonder ruimte voor speculatie- heb ik goed kunnen ontwikkelen dankzij jou zorgvuldige en bruikbare commentaar, welke van heel-veel-rood naar steeds minder rood ging. Ook tijdens mijn coschap reumatologie in het Martini Ziekenhuis nam je altijd de tijd voor feedback en daardoor heb ik in die korte tijd enorm veel van jou kunnen leren, en heb ik mede daardoor voor het vak gekozen.

Hendrikus, vanaf het eerste moment dat ik met jou aan het werk was, heeft jou enthousiasme een aanstekelijke werking op mij gehad. Bij elke labeling en scan was jij er om mij advies te geven, en dankzij jou is het folaat-technetium project een succes geworden. De aanvraag voor de Rubicon beurs heb ik samen met jou geschreven, waarvoor ik je erg dankbaar ben. Helaas is de beurs niet gehonoreerd, maar desondanks beoordeeld als zeer goed.

Clark, de eerste keer dat ik bij jou aan het bureau zat om het folaat project (wat begonnen is als een proefproject) door te spreken weet ik nog goed. Jou doortastende en directe manier van werken heeft voor veel mogelijkheden gezorgd. Ook als ik het even niet zag zitten (bijvoorbeeld na het eerste paper acht keer gesubmit te hebben), wist jij me

altijd weer te motiveren om toch met veel plezier weer verder te gaan. Bedankt voor het vertrouwen en mogelijkheden die je me hebt geboden.

Riemer, als het mogelijk was geweest om nog een co-promotor te hebben dan was jij dat geweest. Bedankt voor jou technische inbreng. Jou deskundigheid op het gebied van nucleaire geneeskunde gecombineerd met je rustige en vriendelijke persoonlijkheid maken jou zeer prettig om mee samen te werken.

René, via jou ben ik bij Clark gekomen en heb ik de kans gekregen om dit project te kunnen doen. Hoewel we niet veel praktisch werk samen gedaan hebben, kon ik altijd rekenen op jou advies op cardiologisch gebied tijdens onze wekelijkse meetings op vrijdagmiddag.
Prof. dr. Cees Kallenberg, uw kennis en ervaring is een grote inspiratiebron voor mij. De verhalen over de wetenschap, maar ook alles daaromheen zullen me altijd bij blijven. Tijdens het congres in Nice dit voorjaar heb ik hier nog van mogen meegenieten.
Prof. dr. Piet Limburg, dankzij jou kritische blik en bruikbare commentaar tijdens de vrijdag ochtend meetings wist ik of ik op de goede weg zat, of het anders aan moest pakken. Ook Alja Stel en Karina de Leeuw hebben daar aan bijgedragen.

De leden van de leescommissie, prof dr. R. Dierckx, prof. dr. P.W. Kamphuisen en prof. dr. G. Pasterkamp, bedankt voor het evalueren van het proefschrift.

Reza, thank you for your help with the scanning and analyzation of the data. In the hours in the basement of the hospital we always had nice conversations, and good coffee during the incubation and scanning.

Nato, I met you during my 1st PhD year, during your internship. Together we wrote a review, which was a very convenient process. You have a warm personality, and it was very pleasant to work with you.

Verder wil ik de mede-auteurs; Go van Dam, Marcel Posthumus, Lodewijk de Groot, Steven Wenker, Andries Smit, Helmy Hinkema, Joop Lefrandt, Jan-Cees Breek, Phillip Low, Bastiaan Wallis de Vries, Jan-Luuk Hillebrands en Niels Harlaar bedanken voor hun bijdrage.

Ook zonder de patiënten en vrijwilligers die hun medewerking verleenden aan de verschillende onderzoeken had dit proefschrift niet tot stand kunnen komen.

Ik wil Gerda, Minke, Johan, Wayel en Theo bedanken voor alle hulp op het lab, en natuurlijk minstens zo belangrijk, alle gezelligheid en bakjes koffie, thee, limonade. In het bijzonder wil ik Berber bedanken. Tijdens mijn coschappen heb jij veel werk gedaan waar ik zelf niet aan toe kwam, kleuringen, PCR en ELISAs, waarvoor ik je erg dankbaar ben. Jou zorgvuldige

werkwijze heeft voor veel betrouwbare resultaten gezorgd, waar ik altijd op verder heb kunnen bouwen. Met de collega's van het diagnostisch lab van de klinische immunologie en de transplantatie immunologie was het altijd gezellig. Bessel en Jetske, bedankt voor de vele racefiets en Nieuw-Zeeland verhalen. Boelo, met de geit gaat het goed. Ook wil ik Kiki en Janny bedanken voor alle administratieve hulp.

Natuurlijk zou dit traject zonder mijn collega PhD studenten veel minder fijn verlopen zijn. Menke, Sarah, Nikola, Sebastian, Alexandre, Birgit, Deena, Suzanne, Fleur, Lucas, Paulina, Niels, Christien, Judith, Qi, Koen en Hans, leuk dat jullie er altijd waren voor een praatje of een bakje koffie. Het was erg fijn om bij gelijkgestemden terecht te kunnen voor alles wat met het onderzoek te maken had maar zeker ook alles daarbuiten.

Mijn mede MD/PhD studenten Marrit en Annemiek, samen met jullie ben ik dit traject in het vierde jaar ingegaan, en ook al zaten we bij verschillende afdelingen, ik heb altijd veel aan jullie gehad om dit traject tot een goed einde te brengen. Leuk dat we twee weken na elkaar promoveren. Hans, bedankt dat ik jou kon opzoeken in de tijd dat je onderzoek deed in New York.

Ik wil Karlijn, Kelly, Irene, Sebastiaan, Martijn en Fia Fia bedanken voor de mogelijkheid om hen te begeleiden tijdens hun stages, dit was erg waardevol. Sofia, gracias por cuidar tan bien de mi en México!

Mijn onderzoekstijd heb ik afgewisseld met coschappen in Heerenveen en Assen. Van beide heb ik heel veel geleerd, waarvoor ik dr. Janneke Zwarts in het bijzonder wil bedanken. Ook mijn mede-co's, zonder jullie had ik niet op dezelfde manier terug kunnen kijken. Hester en Gwen, erg leuk dat we soms nog samen door het Groningse land kunnen fietsen. Tamara, ik kom je zeker nog eens opzoeken in Zwitserland. Nynke, ik heb erg genoten van de tijd dat wij afgewisseld Nynke 1 en Nynke2 waren op afdeling A1 in het WZH. Ook wil ik prof. dr. Hendrika Bootsma, dr. Martha Leijsma en dr. Wilbert Janssen bedanken voor de mogelijkheid om de opleiding tot reumatoloog te volgen in het UMCG en in het Martini Ziekenhuis.

Onderzoek kan natuurlijk ook niet zonder de nodige ontspanning, daarvoor wil ik mijn lieve vriendinnetjes van het 'dispuut de gouden tepel' bedanken voor de vele gezellige avonden die we samen hebben beleefd, de weekendjes weg, de festivals, de sportieve activiteiten. Tsjitske, samen konden we vaak even stoom afblazen van ons promotie werk, en bijna waren we nog op dezelfde dag gepromoveerd. Ik wens jou veel succes in Enschede met je opleiding tot MDL-arts.

Mijn vriendinnen van thuis-thuis, Ilse, Femmie, Marcella en Gonja wil ik bedanken voor

mooie tijd die we hebben gehad, tijdens de etentjes, stapavonden en natuurlijk pinkpop afgelopen zomer.

Ytje, wij zijn al bevriend zolang we het ons kunnen herinneren. Ook al is er nu door de afstand minder tijd om elkaar vaak op te zoeken, het is meteen weer als vanouds als we elkaar weer zien. Bedankt voor de fantastische tijd als student en ver daarvoor, en natuurlijk in Australië en Nieuw-Zeeland, waar we samen gereisd hebben. Drie maanden in een auto leven kun je niet met iedereen, ook al was het dan een grote Ford.

Mijn Heit en Mem wil ik bedanken voor alle steun, vertrouwen en liefde. Ik kijk er altijd enorm naar uit om weer terug te gaan naar Oudega, en op zondag avond bij jullie aan te schuiven voor het diner waar we vervolgens met een goed glas wijn en Franse kaas voor de open haard eindigen. Zonder jullie was me dit niet gelukt. Sybrand, mijn broer(tje), ook al zien we elkaar niet zo vaak, als het er van komt is het altijd gezellig.

Mijn paranimfen Rens en Fiona. Rens, wij kennen elkaar al sinds de middelbare school. Achterin bij Bijlsma in de biologie les was het altijd erg gezellig, waar we uitgebreid onze weekenden doorspraken. Dit werd altijd gedoogd omdat Bijlsma onze verhalen stiekem ook wel interessant vond. Ook tijdens de studententijd hebben we veel mooie momenten beleefd. Ik heb nog steeds erg warme herinneringen aan onze avondjes met goede muziek en wijn op de Haddingedwars.

Fiona, wij hebben elkaar leren kennen als collega's, en kwamen er al snel achter dat we een gezamenlijke hobby hebben; het paardrijden. Daar kwam nog bij dat we het ook nog eens super goed met elkaar kunnen vinden. Bedankt dat ik altijd met je mee mag om op Utopia en Sierra te rijden. Ik heb erg genoten van de lol en de gezelligheid bij de paardrijvierdaagse in Dwingeloo dit jaar. We hebben zelfs de buurvrouw uit de caravan naast ons wakker gekregen met ons gegiechel. De ritten in de Gaasterlandse bossen houden we nog tegoed! Ook je hulp met de multivariate analyses kwam zeer goed van pas. Super dat jullie me bij willen staan tijdens mijn promotie.

Jelmer, samen met jou door het leven gaan is het mooiste wat er is. Tijdens onze studententijd hadden we het soms zo gezellig dat ik het college van 9 uur miste omdat het nog veel te gezellig was met jou in de kroeg. Na mijn MD/PhD sollicitatie was jij de eerste die me belde, misschien was dat al een voorteken. Jij staat altijd voor me klaar, en helpt me bij alles. Die keer dat ik een poster moest inleveren maar mijn SPSS niet werkte en jij voor mij alles hebt uitgerekend is daar een goed voorbeeld van. Ik hoop dat we nog lang samen kunnen genieten van elkaar, en binnenkort van ons nieuwe huis in Groningen.

Nynke

Curriculum Vitae

Nynke Afke Jager werd op 30 augustus 1986 geboren te Oudega (Gaasterlân-Sleat). Na het VWO op het RSG Magister Alvinus te Sneek begon ze, na eerst in Wageningen dierwetenschappen en in Groningen bewegingswetenschappen geprobeerd te hebben, in september 2005 aan de studie geneeskunde. In 2008 kwam ze in aanraking met de wetenschap door een proefproject op de afdeling vaatchirurgie bij Prof. dr. Clark Zeebregts van het Universitair Medisch Centrum Groningen (UMCG). Vervolgens startte ze met een MD/PhD traject in 2010, bij de afdeling Reumatologie en Klinische Immunologie onder leiding van Prof. dr. C. Zeebregts, dr. M. Bijl, dr. H.H. Boersma en dr. J. Westra. Tijdens dit traject werd het wetenschappelijk onderzoek gecombineerd met de co-schappen, welke in de Tjongerschans te Heerenveen en het General Hospital in México gevolgd werden. De semi-arts stage vond plaats op de interne geneeskunde van het Wilhelmina Ziekenhuis te Assen onder leiding van dr. Janneke Zwarts, en de Reumatologie en Klinische Immunologie afdeling van het Martini ziekenhuis te Groningen met supervisie van dr. M. Bijl. In december 2013 haalde ze het arts-examen. Vanaf november 2014 is ze in het kader van de opleiding tot reumatoloog als arts-assistent werkzaam in het Martini Ziekenhuis te Groningen.